REMARKS

Applicant gratefully acknowledges the courtesy shown by Examiner McGaw and Supervisory Patent Examiner (SPE) Housel in the telephonic interview with inventor Christian Münz, Attorney Paul Fehlner and the undersigned of Darby & Darby on March 18, 2005. During the interview, the rejections under 35 U.S.C. § 103(a) were discussed. SPE Housel indicated that the Office would consider claim amendments that clarify the invention, i.e., to recite that the dendritic cell is capable of eliciting an immune response to EBV-infected cells.

Status of the Claims

Claims 35 and 37-39 are pending. Claims 1-34, 36, and 40-45 have been canceled without prejudice as drawn to a non-elected invention. Claim 35 has been amended to include the limitation "capable of eliciting an immune response to EBV-infected cells." Support for this claim amendment can be found in the specification at, for example, page 3, line 25 to page 4, line 1; page 9, lines 8-15; page 10, lines 12-16; page 10, line 30 to page 11, line 4; page 12, lines 2-5; page 33, line 16 to page 34, line 16; and page 35, line 24 to page 44, line 19. Such an immune response is seen through cell lysis in *in vitro* assays (specification, page 35 , line 24 to page 44, line 30).

Claim Rejections Under 35 U.S.C. § 103

The Examiner has maintained the rejection of claims 35 and 37-39 under 35 U.S.C. § 103(a) as obvious over Wong et al., J Immunother. 1998;21(1):32-40 ("Wong") in view of Khanna et al., Immuno Rev. 1999;170:49-64 ("Khanna 1999") and/or Khanna et al., Intern Immun. 1997;9(10):1537-1543 ("Khanna 1997").

The Examiner contends that Wong discloses a method in which a dendritic cell contacted with the EBV latent antigen LMP2a elicited a robust memory cytotoxic T-lymphocyte ("CTL") response.

The Examiner contends that Khanna 1997 and Khanna 1999 disclose that CTLs sensitized with EBNA-1 efficiently recognize EBV-transformed B cells. According to the Examiner, Khanna discloses that "EBNA-1 can be processed via the exogenous pathway ultimately leading to presentation through MHC class II molecules to CD4+ T cells" (Office Action, page 3).

Rickenson was cited to show that there are three predominant EBV latency proteins: LMP1, LMP2 and EBNA-1 (Office Action, page 5).

According to the Examiner, one of ordinary skill in the art would have been motivated to substitute EBNA-1 for LMP-2a in the teachings of Wong because it was widely recognized that EBNA-1 is one of the primary EBV latency-associated antigens. Further, according to the Examiner, priming CTLs with EBNA-1 pulsed dendritic cells "would be an effective strategy to generate a CTL response to EBV-transformed B cells through their expression of EBNA-1 during latency" (Office Action mailed July 16, 2004, pages 4-5). The Examiner concludes that one of ordinary skill in the art would have "expected to be able to make" an EBV-protective dendritic cell by substituting EBNA-1 for LMP2 because the technique for creating antigen-pulsed dendritic cells was well established and Wong discloses methods for making such cells using EBV latency-associated antigens. *Id.* at 5.

This rejection under 35 U.S.C. § 103(a) should be withdrawn because while it is possibly true that one of ordinary skill in the art would have wanted to develop an immune response to cells that express EBNA-1 as a result of EBV infection, the art of record, and the state of the art prior to

this invention, led away from such a modification. Thus, there was no motivation to substitute EBNA-1 for LMP2a in the LMP2a-contacted dendritic cell according to Wong.

A feature of a vaccine for humans is its ability to elicit immunity to a natural pathogen. Eliciting ineffective immune responses in humans borders on the unethical; certainly there is no motivation to do it. Thus, the claimed method of treating human dendritic cells depends on the ability of those cells to elicit an immune response against EBV infected target cells. *See, e.g.,* specification, page 12, lines 2-5 ("The present invention provides ... immunotherapy using EBNA-1 charged dendritic cells to prevent or treat EBV infection.")

With these principles in mind, the Examiner's rejection founders on three basic premises. First, the prior art taught that EBNA-1-specific cytotoxic T-cells would only recognize cells charged with an artificial EBNA-1 construct, that they did not recognize a cell naturally infected with EBV. This fact alone defeats obviousness because it leads away from the invention and shows the failure of others. In particular, based on the prior art teaching, there is no reason to charge *human* dendritic cells with EBNA-1 *ex vivo.* Second, EBNA-1 is expressed inside the cell, so anti-EBNA-1 antibodies are not protective, i.e., they do not clear up the EBV infection. Furthermore, even if EBNA-1 were part of the viral envelope (it is not), there are no viral particles and thus no viral envelope in latent infections. Anti-EBNA-1 antibodies are, as discussed during the interview, merely artifacts. Finally, there is no basis, as the Examiner conceded, for using EBNA-1 as some sort of immunostimulatory molecule to potentiate an immune response to some other antigen.

A dendritic cell contacted with the EBV latent antigen LMP2a may elicit a robust memory cytotoxic T-lymphocyte CTL response able to target, e.g., lyse *in vitro,* EBV-infected cells, but there is no disclosure or suggestion in any of the references that an EBNA-1-contacted dendritic cell

would elicit such a response. The motivation to contact a human dendritic cell with an antigen is to elicit an immune response in a human host. In the case of EBNA-1, the immune response would need to be directed against target cells, which are B-cells infected with Epstein Barr virus (EBV) expressing EBNA-1. The CTLs disclosed in the Khanna publications do not recognize EBV-infected target cells unless those cells are *exogenously stimulated* with recombinant EBNA-1. Of course, an EBV-infected cell is not exposed to exogenous recombinant EBNA-1 in a host.[1] Thus, there would have been no motivation to contact a dendritic cell with EBNA-1 in order to stimulate EBNA-1 specific responder cells, which could not have been expected to produce an EBV-specific immune response because they were found not to recognize natural EBV infected B-cells.

According to Khanna 1997 and Khanna 1999, EBNA-1-specific CTLs do not recognize B cells infected with EBV as the infection occurs in nature. For example, Khanna 1999 discloses that in an "[e]xtensive analysis of CTL responses in a large panel of healthy virus carriers....no reactivity towards EBNA1 was detected..." (Khanna 1999, page 51) (emphasis added). Khanna 1999 states that the glycine-alanine repeat sequences within EBNA1 have an inhibitory effect on endogenous processing of EBNA1 through the class I pathway, which "may on occasion be overridden *in vivo,* since...EBNA1-specific CTLs have been detected in healthy virus carriers. *Interestingly, these EBNA1-specific CTLs can only recognize LCLs to which recombinant EBNA1 protein has been supplied exogenously*" (Khanna 1999, page 51) (emphasis added). Thus, Khanna 1999 teaches that CTLs would only recognize target cells to which a recombinant EBNA-1 construct has been supplied. Similarly, Khanna 1997 discloses "that although *EBV-infected, HLA-DR1-positive B cells*

[1] Furthermore, it would be absurd to introduce modified EBNA-1 into the host to elicit an immune response to EBV-infected cells since there is no way to ensure that the EBV-infected cells would preferentially take up and present the modified EBNA-1 construct. Thus, the result would be generalized inflammation with no particular effect on EBV infection.

*were **not** recognized by the EBNA1-specific CTL clone DM2*, exogenous sensitization of these cells with either the AcEBNA1 [a baculovirus construct, which expresses EBNA1, *see* Khanna 1997 at 1538] protein or the E1NX protein [truncated EBNA1 antigen, *see* Khanna 1997 at 1538] completely restored the immune recognition by these CTL" (Khanna 1997 at 1539, 2nd column).

In contrast, the instant specification discloses EBNA-1 specific T-cell recognition of EBV-infected B-cells, as the infection occurs in nature. CD4+ T cell lines recognized dendritic cells that were infected with a vaccinia virus EBNA-1 construct (specification, page 42, lines 16-17), and EBNA-1 specific T-cells recognized (specification, page 43, lines 10-11) and killed (specification, page 44, lines 12-19) EBV-transformed B-cells. These findings have been confirmed by independent laboratories subsequent to the filing of the instant application. For example, Fu *et al.* disclose that: "vaccination of mice with EBNA1 peptide-loaded DCs can elicit CD4+ T cell responses. These EBNA1-specific CD4+ T cells recognized peptide-pulsed targets as well as EBNA1-expressing tumor cells and were necessary and sufficient for suppressing tumor growth *in vivo*" (Fu *et al.*, J Clin Invest. 2004;114(4):542-550, abstract; attached as Exhibit 1).

To summarize, none of the references disclose or suggest that EBNA-1-specific responder T-cells result in an immune response to EBV-infected B-cells. The Khanna references disclose T-cell recognition of EBV-infected B-cells *only if* the cells are stimulated with exogenous recombinant EBNA-1. EBV infection in nature does not involve stimulation by exogenous, recombinant protein. The reason to load a human dendritic cell *ex vivo* with an antigen is to use the loaded dendritic cell to produce an immune response effective to treat to the condition (infection) associated with the antigen. According to the references, EBNA-1 antigen does not result in an immune response effective to treat a natural EBV infection. Thus, one of ordinary skill in the art would have had no

motivation to remove a dendritic cell from a human and load it with an antigen (EBNA-1). The motivation to load a dendritic cell with EBNA-1 was provided only upon the discovery disclosed in the present specification that EBNA-1-specific T-cells lyse EBV-transfected B-cells.

The Examiner contends, in a statement that is not supported by the references, that an EBNA-1 loaded dendritic cell "would stimulate an antibody response to EBNA1, which would constitute an immune response against EBV" (Office Action, page 6). This is not the case as EBNA-1 is expressed inside the cell. *See* Reedman, B.M. and Klein, G., Int J Cancer. 1973; 11, 499-520 (first proposing that this nuclear antigen be referred to as "EBNA," see page 517) (copy attached as Exhibit 2); Kieff, E. and Rickinson, A., Fields Virology, (D.M. Knipe and P. M. Howley eds., 2001) at 2529 (copy attached as Exhibit 3). Thus, anti-EBNA-1 antibodies do not elicit an immune response to EBV-infected cells. *See* Kieff and Rickinson at 2583 (Exh. 3). Further, EBNA-1 is not part of the viral envelope and, therefore, anti-EBNA-1 antibodies could not elicit an immune response to Epstein Barr viral particles. *See* Kieff and Rickinson at 2583 (Exh. 3). Even if the antibodies could elicit such an immune response, there are no viral particles in latent infections.

The Examiner also asserts in an unsupported statement that "such a dendritic cell would produce chemokines, cytokines and IFN-α/ß ... [which] would presumably enhance the CTL response against other EBV peptides such as LMP1 and LMP2" (Office Action page 6). This is speculative at best. The references do not disclose or suggest such a role for EBNA-1 charged dendritic cells. The Examiner has not met his burden to show that this role for an EBNA-1 charged dendritic cell was known in the art at the time of filing, or that if it was known, it would have suggested the invention. Subsequent to filing, it was established that cytokines clearly did not have such a role: "[W]hile cytokine expression reflects the differentiation and activation state of the

[CD4+ T-cell] clones [reactive to EBNA-1] and helps to classify them, the secreted cytokines themselves are not likely to be essential effector mechanisms" (Nikiforow *et al.*, J Virol. 2003;77(22):12088-12104; 12099-12100, attached as Exhibit 4).

In a third unsupported statement, the Examiner contends that the motivation to combine the references was present because "CD4+ and CD8+ EBNA1-specific CTL populations would be increased by the EBNA1 contacted cells," which would be "desirable." (Office Action, pages 6-7). As discussed above, the references do not provide any motivation to contact a human dendritic cell with EBNA-1 because there was no disclosure or suggestion that it would expand a CD4+ population, much less that expanding a CD4+ population would result in an immune response to naturally infected EBV cells. Nor do the references disclose or suggest that expanding a CD8+ population would result in such an immune response. In fact, it was subsequently shown that CD8+ cells do not have this effect: "EBNA1 positive tumor cells [are] invisible to the host CD8+ T cells" (Fu *et al.*, page 542, Exh. 1).

The Examiner contends that the dendritic cells disclosed in the Khanna references, in combination with Wong, render the instant claims obvious because: "[i]t is also possible that the CD4+ T cells may be directly involved in the lysis of EBNA1 expressing cells." No reference, or combination of references, disclosed or suggested T-cell recognition and lysis of EBV-infected cells as they exist in a human. This was first disclosed in the instant specification. For example, "[t]he nuclear antigen EBNA-1 is repeatedly recognized by CD4+ T cells from healthy adults. The CD4+ T cells are capable of proliferation, cytokine secretion and cytolytic activity" (specification, page 44, lines 20-23). This discovery, which was made as part of the present invention and is not in the prior

art, leads to the claimed invention, to charge a human dendritic cell *ex vivo* with EBNA-1. *See,* specification, page 35, line 24 to page 44, line 30.

Accordingly, the rejections under 35 U.S.C. § 103(a) should be withdrawn.

Conclusion

No new matter has been added. All of the pending claims in this application are believed to be in condition for allowance. Entry and consideration of these amendments and remarks are therefore respectfully requested.

Respectfully submitted,

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By _____

Paul M. Zagar, M.D.
Registration No.: 52,392
DARBY & DARBY P.C.
P.O. Box 5257
New York, New York 10150-5257
(212) 527-7700
(212) 753-6237 (Fax)
Attorneys/Agents For Applicant

Exhibit 1

Research article

Critical role of EBNA1-specific CD4+ T cells in the control of mouse Burkitt lymphoma in vivo

Tihui Fu, Kui Shin Voo, and Rong-Fu Wang

The Center for Cell and Gene Therapy and Department of Immunology, Baylor College of Medicine, Houston, Texas, USA.

CD4+ T cells play important roles in orchestrating host immune responses against cancer and infectious diseases. Although EBV-encoded nuclear antigen 1–specific (EBNA1-specific) CD4+ T cells have been implicated in controlling the growth of EBV-associated tumors such as Burkitt lymphoma (BL) in vitro, direct evidence for their in vivo function remains elusive due to the lack of an appropriate experimental BL model. Here, we describe the development of a mouse EBNA1-expressing BL tumor model and the identification of 2 novel MHC H-2I-A^b–restricted T cell epitopes derived from EBNA1. Using our murine BL tumor model and the relevant peptides, we show that vaccination of mice with EBNA1 peptide–loaded DCs can elicit CD4+ T cell responses. These EBNA1-specific CD4+ T cells recognized peptide-pulsed targets as well as EBNA1-expressing tumor cells and were necessary and sufficient for suppressing tumor growth in vivo. By contrast, EBNA1 peptide–reactive CD8+ T cells failed to recognize tumor cells and did not contribute to protective immunity. These studies represent what we believe to be the first demonstration that EBNA1-specific CD4+ T cells can suppress tumor growth in vivo, which suggests that CD4+ T cells play an important role in generating protective immunity against EBV-associated cancer.

Introduction

EBV is a human gammaherpesvirus with tropism for B cells and has been associated with several types of malignant tumors, including Burkitt lymphoma (BL), post-transplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma (NPC), and Hodgkin disease (HD) (1–3). Although a subset of genes is responsible for the growth-transforming function of EBV, *EBV-encoded nuclear antigen 1* (*EBNA1*) is the only viral gene that is regularly detected in all EBV-associated tumors (BL, NPC, PTLD, and HD) and is required for the long-term persistence of EBV as well as the pathogenesis of EBV-associated cancers (3–5). Increasing evidence indicates that T cell responses to EBNA1 are important in controlling EBV infection (3, 6, 7), which suggests that EBNA1 is an important target for immunotherapy of EBV-associated malignancies.

However, the presence of the glycine and alanine repeat (GAr) domain within EBNA1 not only blocks its proteasomal degradation for the MHC class I antigen processing pathway, but also inhibits its own mRNA translation (8–11). Although we have recently identified a naturally processed HLA-B8–restricted epitope from EBNA1 (12), the overall capacity of MHC class I antigen processing and presentation in BL cells is significantly impaired, making EBNA1-positive tumor cells invisible to the host CD8+ T cells. By contrast, the EBNA1 protein can be normally processed and presented through the MHC class II processing pathway and elicits consistent CD4+ T cell immune response (7, 13–16). As a result, several MHC class II–restricted EBNA1 peptides have been identified (13, 17–19). These observations imply that EBNA1-specific CD4+ T cells may play a role in controlling tumor growth in vivo. However, due to the lack of a reliable animal model for EBV-associated tumors, the role of EBNA1-specific CD4+ T cells in antitumor immunity in vivo remains to be defined.

In this article, we describe the establishment of a murine BL model and the identification of EBNA1-derived T cell peptides for recognition by CD4+ T cells. We show that immunization of mice with EBNA1-derived T helper peptides can elicit potent CD4+ T cell responses and inhibit tumor growth following subsequent tumor challenge. More importantly, EBNA1-specific CD4+ T cells, but not CD8+ T cells, contributed to the observed antitumor immunity. These results suggest that EBNA1-specific CD4+ T cell response elicited by DC loaded with EBNA1 peptide (DC/EBNA1 peptide) vaccination plays an important role in inhibiting in vivo growth of EBNA1-expressing B6-BL tumor cells.

Results

Establishment and characterization of BL cell lines. The B6-BL murine cell line, initially generated from a human *Igλ-MYC*–transgenic mouse, shares many characteristics with human BL (20). The B6-BL cell line expressing EBNA1 (B6-BL/EBNA1) was generated from *Igλ-MYC* × EBNA1 double-transgenic mice, but the EBNA1 expression level could not be detectable by Western blot analysis with an EBNA1-specific antibody (data not shown). To make certain that EBNA1 was properly expressed in the murine BL cells, we successfully transduced B6-BL cells with a retroviral vector encoding EBNA1-GFP and designated the resultant cell line B6-BL/EBNA1-GFP. Expression of *EBNA1-GFP* fusion gene allowed us to monitor EBNA1 expression in the cells. B6-BL cell line expressing GFP (B6-BL/GFP) served as a control. EBNA1 expression in the B6-BL/EBNA1-GFP tumor cells was confirmed by Western blot analysis (Figure 1A). Further characterization of the B6-BL/EBNA1-GFP and B6-BL/GFP cell lines by FACS analysis with a panel of anti-

Nonstandard abbreviations used: Burkitt lymphoma (BL); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT); EBV-encoded nuclear antigen 1 (EBNA1); GAr-deleted-EBNA1 (GAr-del-EBNA1); glycine and alanine repeat (GAr); Hodgkin disease (HD); nasopharyngeal carcinoma (NPC); post-transplant lymphoproliferative disorder (PTLD).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 114:542–550 (2004). doi:10.1172/JCI200422053.

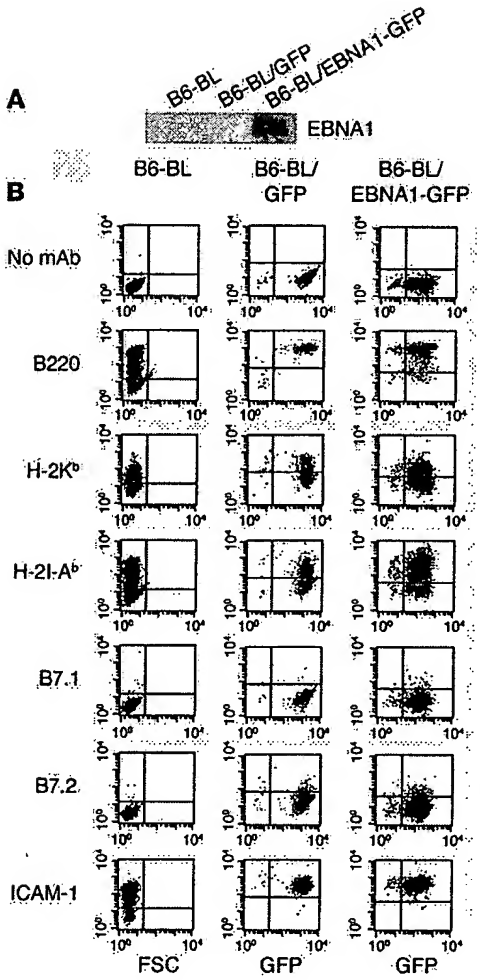

Figure 1
Generation and characterization of an EBNA1 expressing BL cell line. (**A**) BL cell lines were transduced to express the full-length *EBNA1-GFP* fusion gene. Expression of GFP served as a control. The expression of full-length EBNA1 protein in the B6-BL/EBNA1-GFP cells was determined by Western blot analysis using anti-EBNA1 mAb (1H4). (**B**) Expression patterns of cell-surface molecules and GFP on these tumor cell lines were analyzed by FACS, combined with a panel of mAb's, which are labeled on the left. FSC, forward scatter.

bodies revealed uniform expression of B220 B cell marker and of H-2K^b, I-A^b, and ICAM-1 molecules but little or no expression of CD80 (B7.1) or CD86 (B7.2) (Figure 1B). Thus, the B6-BL/EBNA1-GFP line was considered to closely resemble human EBNA1-positive BL cells, although some human BL cells do not express MHC class I and ICAM-1 molecules.

Immunogenicity of B6-BL/EBNA1-GFP cells. To test whether the expression of EBNA1-GFP or GFP in B6-BL cells might affect tumor immunogenicity as determined by growth properties, we examined the proliferation of BL cell lines both in vitro and in vivo. As shown in Figure 2A, the B6-BL, B6-BL/GFP, and B6-BL/EBNA1-GFP cells exhibited similar or identical growth activities in vitro by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The immunogenicity of B6-BL/EBNA1-GFP and B6-BL/GFP was assessed in vivo by subcutaneously injecting tumor cells into syngeneic B6 mice in different doses (from 2.5×10^5 to 1×10^6 tumor cells). All injections resulted in tumor growth, which became detectable 6–12 days after inoculation, depending on the number of tumor cells injected (data not shown). In a subsequent experiment, we subcutaneously injected mice with 5×10^5 tumor cells and measured tumor growth every 2 days. All 3 tumor cell lines had similar growth properties in vivo (Figure 2B), which suggests that neither EBNA1 nor GFP expression in B6-BL cells affected tumor cell immunogenicity.

Identification of EBNA1-specific T cell epitopes. Having established a BL mouse model with characteristics similar to human BL, we sought to identify EBNA1-derived T cell epitopes presented by murine MHC class II molecules. We first evaluated whether EBNA1 could stimulate T cell responses in B6 mice immunized with full-length or truncated forms of EBNA1 (GAr-deleted EBNA1, or GAr-del-EBNA1). T cells from splenocytes of the immunized mice were stimulated in vitro with 10 EBNA1 peptides, as previously described (19). After 6 days of stimulation, T cells from the draining lymph nodes of B6 mice vaccinated with the full-length EBNA1 protein showed strong reactivity against the EBNA1-$P_{607\text{-}619}$ peptide as compared with results for the 9 remaining peptide candidates (Figure 3A, upper panel). Similar results were obtained with T cells derived from B6 mice immunized with GAr-del-EBNA1 protein (Figure

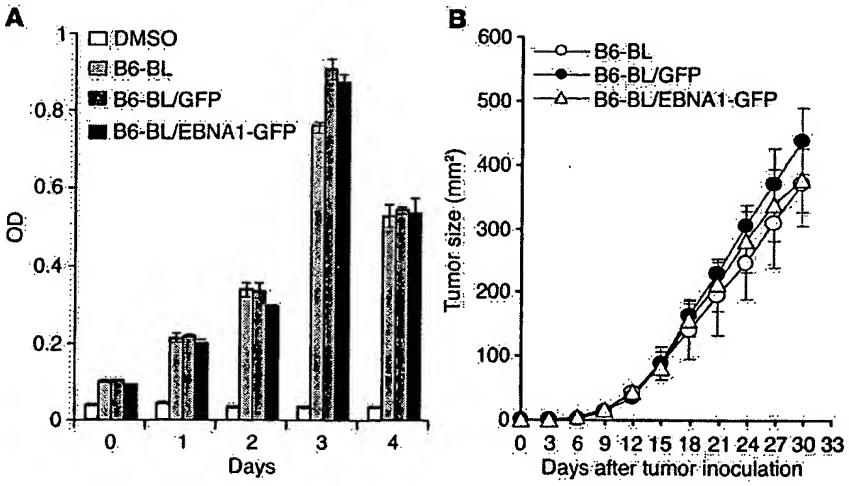

Figure 2
Immunogenicity of BL cells. (**A**) Comparison of in vitro growth of BL cell lines expressing GFP or EBNA1-GFP using MTT assay. Data represent mean ± SEM of triplicate cultures. There were no significant differences in tumor growth among the cell lines. (**B**) The growth of tumor cell lines in vivo. Mice were subcutaneously injected with 5×10^5 of B6-BL, B6-BL/GFP, or B6-BL/EBNA1-GFP tumor cells at day 0. Tumor size was recorded in mm² every 3 days. The results, reported as means ± SEM for 5 mice, indicate that neither EBNA1 nor GFP affected the immunogenicity of B6-BL tumor cells.

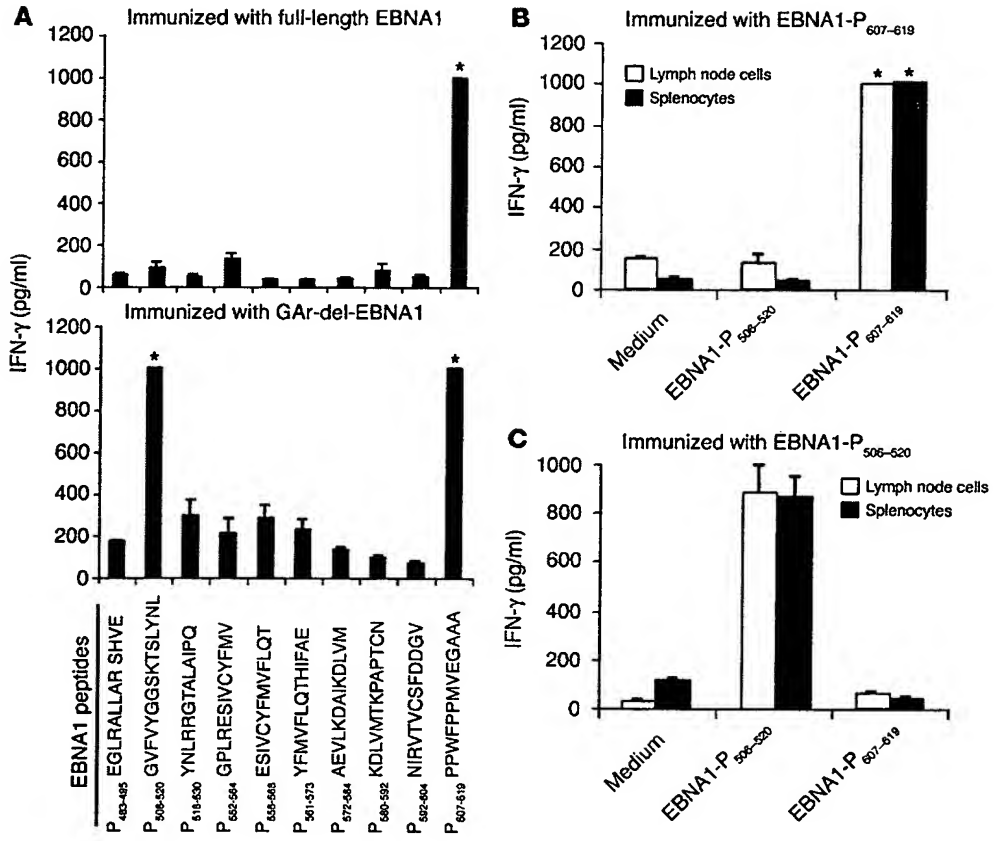

Figure 3

Identification of new EBNA1-specific T cell epitopes presented by murine I-A^b molecules. (**A**) EBNA1 peptides recognized by T cells from the immunized mice. B6 mice were immunized with 50 μg of full-length EBNA1 protein per mouse (upper panel) or GAr-del-EBNA1 (lower panel) in CFA. Eleven days later, T cells from draining lymph nodes of the mice were prepared, and 5 × 10^5 cells were stimulated in vitro in the presence of 10 μM of various synthetic peptides derived from EBNA1. After overnight culturing, the supernatants were tested for IFN-γ release by ELISA. (**B** and **C**) Generation of EBNA1-specific T cells after vaccination of mice with the newly identified T cell peptides. Both splenocytes (black bars) and lymph node cells (white bars) from mice immunized with 100 μg/mouse of EBNA1-$P_{607-619}$ (**B**) or EBNA1-$P_{506-520}$ (**C**) were stimulated in vitro with the corresponding as well as control peptides, and IFN-γ secretion was determined. Asterisks indicate that the readings at OD 450 nM for IFN-γ release were higher than those at the highest concentration (1,000 pg/ml) of the IFN-γ standards.

3A, lower panel). Besides the EBNA1-$P_{607-619}$ peptide, T cells from B6 mice immunized with the GAr-del-EBNA1 protein recognized a new EBNA1-$P_{506-520}$ peptide (Figure 3A, lower panel). To further test the immunogenicity and specificity of the peptides, we immunized mice with either EBNA1-$P_{607-619}$ or EBNA1-$P_{506-520}$ peptide. T cells from B6 mice immunized with EBNA1-$P_{607-619}$ recognized the same EBNA1-$P_{607-619}$ peptide, but not the EBNA1-$P_{506-520}$ peptide (Figure 3B). Conversely, T cells from B6 mice immunized with EBNA1-$P_{506-520}$ recognized the EBNA1-$P_{506-520}$ peptide, but not the EBNA1-$P_{607-619}$ peptide (Figure 3C). Taken together, these results suggest that while both EBNA1-$P_{506-520}$ and EBNA1-$P_{607-619}$ are capable of stimulating EBNA1-specific T cell responses, only the EBNA1-$P_{607-619}$ peptide is naturally processed and presented to T cells. Hence, all further studies to elucidate the role of EBNA1-specific T cells in the induction of antitumor immunity were conducted with this antigenic EBNA1-$P_{607-619}$ peptide.

Induction of both CD4⁺ and CD8⁺ T cell responses by DC/EBNA1-$P_{607-619}$ peptide vaccination. Since DCs are the most effective APCs for the induction of T cell-mediated immune responses (21), we next asked whether T cell responses could be elicited by DC/peptide vaccination. DCs generated from the bone marrow cells of B6 mice and pulsed with the EBNA1-$P_{607-619}$ peptide were used to immunize syngeneic B6 mice. Spleen cells isolated from these mice were stimulated in vitro with the EBNA1-$P_{607-619}$ peptide for 6 days and tested against 293I-A^b cells pulsed with EBNA1-$P_{607-619}$ peptide or the control EBNA1-$P_{572-584}$ peptide. IFN-γ release from T cells was not observed when T cells were stimulated with 293I-A^b cells alone or after pulsing with a control EBNA1-$P_{572-584}$ peptide. By contrast, significant amounts of IFN-γ were detected in the supernatants of T cells stimulated with 293I-A^b cells pulsed with the EBNA1-$P_{607-619}$ peptide (Figure 4A), which suggests that these $CD4^+$ T cells are capable of recognizing the EBNA1-$P_{607-619}$ peptide. To test whether EBNA1-$P_{607-619}$–specific $CD8^+$ T cells were elicited, we used EL-4, a murine T lymphoma cell line that expresses MHC class I (K^b) but not class II molecules. Little or no IFN-γ release from T cells was detectable after coculturing of T cells with EL-4 alone or EL-4 pulsed with EBNA1-$P_{572-584}$ control peptide. However, T cells from the immunized mice could respond to EL-4 cells pulsed with the EBNA1-$P_{607-619}$ peptide (Figure 4B). To further confirm these results, we performed intracellular cytokine staining of T cells after stimulation with EBNA1 peptides. As shown in Figure 4C, $CD4^+$ T cells from the EBNA1-$P_{607-619}$ peptide–immunized mice could produce IFN-γ upon stimulation with the same peptide. The percentage of T cells double positive for CD4 and IFN-γ was 0.94, compared with 0.02% after stimulation with the control EBNA1-$P_{572-584}$ peptide. Similarly, EBNA1-$P_{607-619}$ stimulation resulted in 1.79% of the T cells becoming double positive for CD8 and IFN-γ, compared with 0.03% following stimulation with the control EBNA1-$P_{572-584}$ peptide. These results suggest that EBNA1-$P_{607-619}$ peptide vaccination activates both $CD4^+$ and $CD8^+$ T cells.

Endogenous processing and presentation of EBNA1-$P_{607-619}$ peptide. Although T cell responses against peptides could be induced from human PBMCs or mice, in many cases the T cells fail to recognize antigen-expressing targets or tumor cells due to either the low affin-

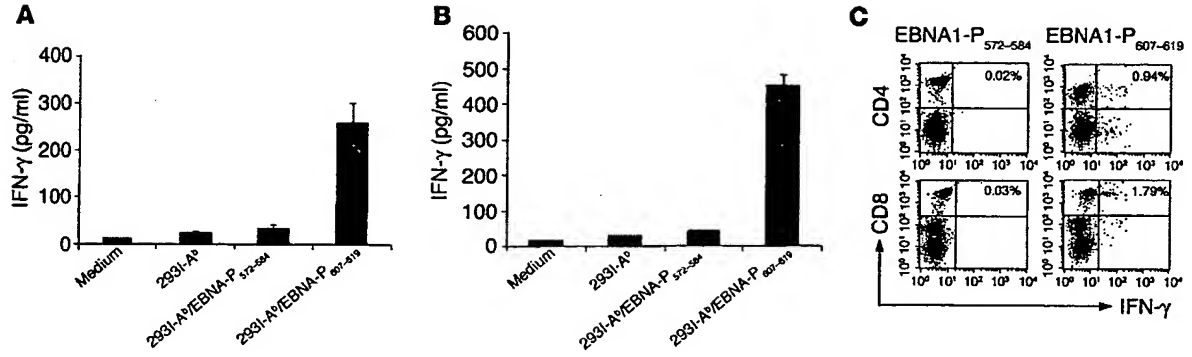

Figure 4

Characterization of EBNA1-$P_{607-619}$ peptide–specific CD4$^+$ and CD8$^+$ T cells. (**A**) Recognition of EBNA1-$P_{607-619}$ peptide by CD4$^+$ T cells. Mice were immunized with EBNA1$P_{607-619}$ peptide loaded onto bone marrow cell–derived DCs. After 2 weeks, splenocytes were prepared and stimulated in vitro with the peptide for 6 days, then tested against the same peptide-pulsed 293I-A^b cells for T cell recognition. EBNA1-$P_{572-584}$ peptide was used as a control. Data are means ± SEM of triplicate cultures. (**B**) Recognition of peptide-pulsed EL-4 target cells by CD8$^+$ T cells. (**C**) Intracellular staining of EBNA1 peptide–specific T cell responses. For intracellular IFN-γ staining, splenocytes were stimulated in vitro with EBNA1$P_{607-619}$ or EBNA1-$P_{572-584}$ (control) peptide overnight and stained with anti-CD4 and anti-CD8 mAb, respectively, followed by intracellular IFN-γ staining. The double-positive T cells were identified by FACS analysis. The percentage of double-positive cells is given in the upper right of each panel.

ity of the T cells or to the inability of tumor/target cells to present naturally processed peptides on their surface. Thus, we asked whether EBNA1-$P_{607-619}$-specific T cells could recognize target cells expressing EBNA1. 293I-A^b cells and a murine prostate tumor cell line expressing H-2Kb but not I-A^b (RM1) were transfected with plasmid DNAs carrying the full-length or GAr-del-*EBNA1* and used to stimulate T cells from EBNA1-$P_{607-619}$–immunized mice. As shown in Figure 5, little or no T cell activity was detected after stimulation with target cells transfected with the empty vector. However, T cells strongly recognized 293I-A^b cells transfected with either GAr-del-EBNA1 or full-length EBNA1, whereas only weak or negligible T cell activity was observed against RM1 cells transfected with the same constructs, which suggests that the EBNA1-specific MHC class II–restricted EBNA1-$P_{607-619}$ peptide is naturally processed and presented to T cells, while the MHC class I–restricted peptides are not naturally processed.

Inhibition of tumor growth by EBNA1-$P_{607-619}$ immunization. We next tested whether immunization of mice with DCs pulsed with EBNA1-$P_{607-619}$ could inhibit tumor growth upon tumor challenge. B6 mice were immunized by a single i.v. injection of 3×10^5 syngeneic DCs loaded with the EBNA1-$P_{607-619}$ peptide or a control EBNA1-$P_{572-584}$ peptide. Two weeks later, they were challenged by subcutaneous injection of B6-BL/EBNA1-GFP cells or control cell lines B6-BL and B6-BL/GFP. Immunization of mice with DC/EBNA1-$P_{607-619}$ peptide resulted in significant inhibition of B6-BL/EBNA1-GFP tumor growth but did not affect the growth of B6-BL or B6-BL/GFP tumor cells, which suggests that antitumor immunity is specific for EBNA1-expressing tumor cells (Figure 6A). Furthermore, immunization of mice with DC/EBNA1-$P_{572-584}$ control peptide failed to inhibit the growth of B6-BL/EBNA1-GFP tumor cells. Similar results were obtained in subsequent experiments that included additional controls for the specificity of antitumor immunity (Figure 6B). These findings indicate that DC/ EBNA1-$P_{607-619}$ immunization elicited antigen-specific immunity, leading to significant inhibition of the growth of B6-BL/EBNA-GFP tumor cells.

Tumor reactivity of EBNA1-$P_{607-619}$–specific CD4$^+$ T cells. To determine whether the EBNA1-$P_{607-619}$–elicited T cells were responsible for the observed inhibition of tumor growth in vivo, we first tested whether T cells elicited from DC/EBNA1-$P_{607-619}$–immunized mice were capable of recognizing B6-BL/EBNA1-GFP tumor cells. T cells were generated from the immunized mice and then tested against B6-BL, B6-BL/GFP, B6-BL/EBNA1-GFP, and tumor cells expressing cancer-testis antigen NY-ESO-1–fused GFP (RM1/NY-ESO-GFP tumor cells). T cells strongly recognized B6-BL/EBNA1-GFP tumor cells but did not respond to B6-BL, B6-BL/GFP, or RM-1/NY-ESO-1-GFP, as determined by IFN-γ release in ELISA and ELISPOT assay (Figure 7, A and B), which suggests that T cells were specific for tumor cells expressing EBNA1 but not GFP. We next determined the relative contribution of CD4$^+$ and CD8$^+$ T

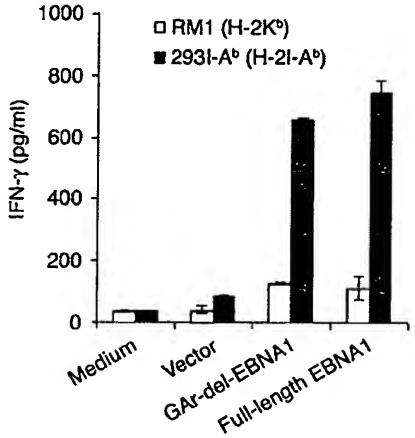

Figure 5

Endogenous presentation of EBNA1-$P_{607-619}$ epitope for T cell recognition. T cells from the immunized mice were tested for their ability to recognize 293I-A^b (black bars) and RM1 (white bars) cells transfected with vectors encoding full-length EBNA1, GAr-del-EBNA1 cDNA, or an empty vector. T cells recognized I-A^b–positive 293 cells transfected with vectors encoding full-length EBNA1 or GAr-del-EBNA1 cDNA, but not with empty vector; they did not recognize I-A^b–negative RM1 cells transfected with the full-length or GAr-del-EBNA1 cDNAs.

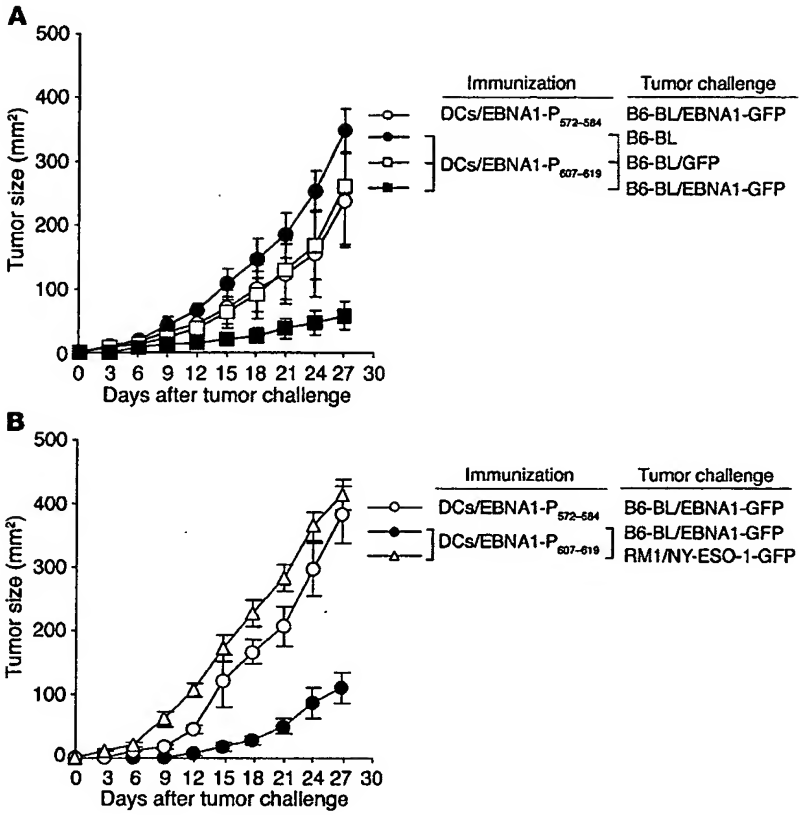

Figure 6
Inhibition of BL tumor growth by EBNA1-specific T cells in vivo. **(A)** Inhibition of tumor growth in DC/EBNA1-vaccinated mice. Two weeks after immunization with DCs pulsed with EBNA1-$P_{607-619}$ peptide or a control EBNA1-$P_{572-584}$ peptide, mice were challenged with 5×10^5 B6-BL/EBNA1-GFP cells or control tumor cell lines B6-BL, B6-BL/GFP. Tumor growth was measured every 2 days. Growth of B6-BL/EBNA1-GFP tumor cells was significantly inhibited in the mice immunized with DC/EBNA1-$P_{607-619}$ peptide compared with other control groups ($P = 0.0174$). **(B)** Specific suppression of B6-BL/EBNA1-GFP tumor cells. To further demonstrate specific inhibition of EBNA1-expressing tumor cells, we immunized mice with DCs loaded with EBNA1-$P_{607-619}$ peptide or a control peptide and challenged them with B6-BL/EBNA1-GFP or RM1/NY-ESO-1-GFP cells. Tumor size was measured every 2 days. Data are means ± SEM ($P = 0.0295$).

cell responses to B6-BL/EBNA1-GFP cells through T cell assay in the presence of anti-CD4 or anti-CD8 mAb. As shown in Figure 7C, IFN-γ release by T cells was not affected by the addition of anti-CD8 mAb's but was markedly reduced when anti-CD4 mAb's were added. These results suggest that EBNA1-specific $CD4^+$ T cells contributed to the inhibition of B6-BL/EBNA1-GFP tumor growth observed in vivo.

$CD4^+$ T cells are responsible for the inhibition of BL growth in vivo. To gain direct evidence for the role of $CD4^+$ T cells in antitumor immunity, we immunized CD4 KO, CD8 KO, and wild-type mice (B6) with DC/EBNA1-$P_{607-619}$. Two weeks later, these mice were challenged with 5×10^5 viable B6-BL/EBNA1-GFP tumor cells. As shown in Figure 8A, tumors grew rapidly in mice immunized with DC/EBNA1-$P_{572-584}$ control peptide and challenged with B6-BL/EBNA1-GFP cells. However, in both wild-type and CD8 KO mice immunized with DC/EBNA1-$P_{607-619}$, B6-BL/EBNA1-GFP tumor growth was significantly inhibited. By contrast, B6-BL/EBNA1-GFP tumor growth was not affected in CD4 KO mice. In fact, tumor growth in these mice was even faster than in wild-type mice immunized with a control EBNA1-$P_{572-584}$ peptide. Similar results were obtained in several independent experiments (data not shown).

To obtain further evidence for the role of EBNA1-specific $CD4^+$ T cells in the control of BL development, MHC class I (deficient in $CD8^+$ T cells) and class II (deficient in $CD4^+$ T cells) KO mice were immunized with DC/EBNA1-$P_{607-619}$ peptide and challenged with B6-BL/EBNA1-GFP. As shown in Figure 8B, tumor growth was remarkably inhibited in $CD4^+$ T cell–intact class I KO mice but not in $CD4^+$ T cell–deficient class II mice. We also performed T cell depletion experiments by intraperitoneal injection of the immunized mice with anti-CD4 and anti-CD8 mAb 1 day before tumor challenge and on days 1, 3, and 7 after challenge. Mice depleted of $CD8^+$ T cells retained the ability to control tumor growth, while those depleted of $CD4^+$ T cells failed to inhibit tumor growth (Figure 8C). Taken together, our results strongly suggest that EBNA1-$P_{607-619}$–specific $CD4^+$ T cells, but not $CD8^+$ T cells, are responsible for the observed antitumor immunity in vivo.

Discussion

Transgenic mice generated from a human *Igλ-MYC* fusion construct developed transgenic lymphoma with a pathology similar to BL (20). Because all BL tumors carry a reciprocal chromosomal translocation between immunoglobulin loci and *MYC* gene, reconstitution of B6-BL tumor cells (containing a human *Igλ-MYC* transgene) with EBNA1 mimics human EBV-associated BL. Human EBV-positive BL cells express EBNA1 but small or undetectable levels of other viral antigens. Thus, the new mouse EBNA1-expressing B6-BL tumor model established in this study reiterates many characteristics of human EBV-positive BL. Coexpression of *MYC* and EBNA1 in double-transgenic mice has been reported to promote lymphomagenesis (22). Since EBV does not infect murine B cells, it is difficult to generate such a model that completely recapitulates human EBV-positive BL at the present time. The purpose of this study was to establish an EBNA1-expressing B6-BL tumor model that would allow us to define the role of EBNA1-specific $CD4^+$ T cells in T cell–mediated antitumor immunity in vivo. Interestingly, EBNA1 expression in B6-BL tumor cells did not change the immunogenicity of B6-BL/EBNA1 cells compared with the growth property of the parental B6-BL cell line. This may be explained by the fact that the GAr domain in EBNA1 not only inhibits the translation of its own mRNA, but also blocks the degradation of

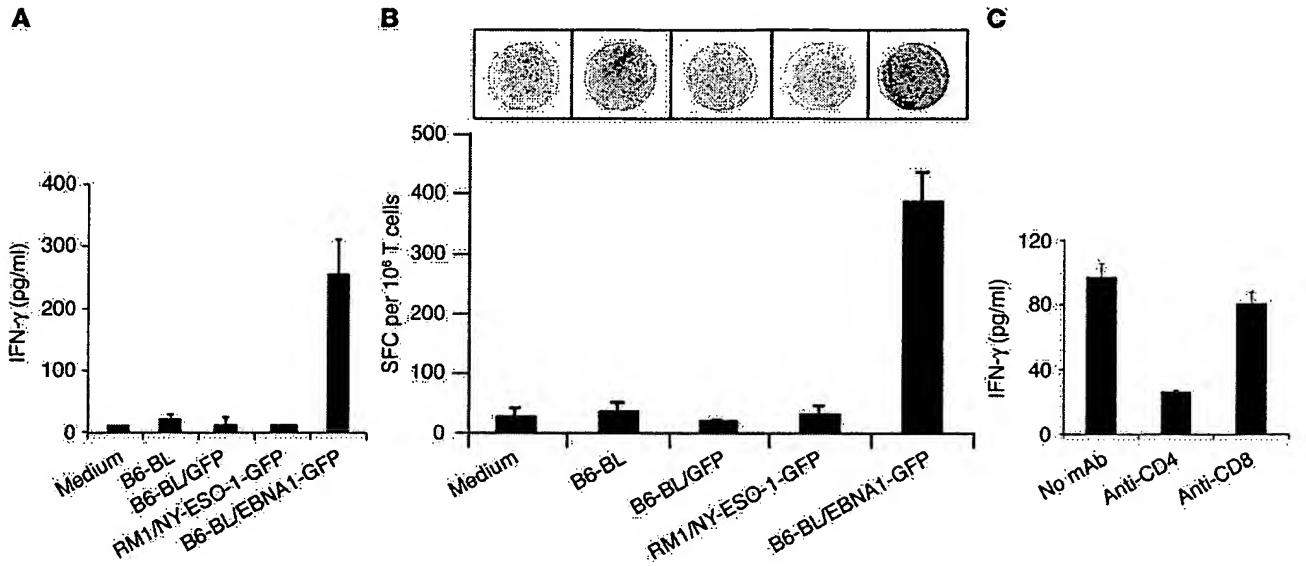

Figure 7

Correlation of T cell activity with the inhibition of BL. (**A**) T cell recognition of B6-BL/EBNA1-GFP cells. T cells from EBNA1-$P_{607-619}$ peptide–immunized mice were tested against a panel of tumor cell lines for tumor reactivity. IFN-γ release was determined by ELISA. (**B**) Tumor reactivity of T cells from the immunized mice as determined by ELISPOT. The antigen-irrelevant tumor cell line RM1/NY-ESO-1-GFP was used as a control. SFC, spot forming cells. (**C**) Identification of the T cell population ($CD4^+$ or $CD8^+$) responsible for tumor reactivity. We tested the ability of T cells to respond to B6-BL/EBNA1-GFP tumor cells in the presence of anti-CD4 and anti-CD8 mAb. Addition of anti-CD4 mAb abolished tumor cell recognition, while the presence of anti-CD8 mAb had no effect.

EBNA1 by proteasomes, thus significantly reducing its capacity to generate MHC class I–restricted peptides (8, 11).

To further assess antigen-specific antitumor immunity in the B6-BL/EBNA1 animal model, we identified two EBNA1-derived T cell epitopes that are presented by murine MHC I-A^b molecules to $CD4^+$ T cells and are capable of eliciting anti-EBNA1 immune responses in EBNA1-immunized B6 mice. Of particular interest is that the EBNA1-$P_{607-619}$ peptide could be processed and presented by murine I-A^b molecules. Although the EBNA1-$P_{607-619}$ peptide induced both $CD4^+$ and $CD8^+$ T cell responses against the peptide-pulsed target cells (Figure 4), $CD8^+$ T cells failed to recognize RM1 cells expressing EBNA1, which suggests that MHC class I–restricted EBNA1 epitopes are not naturally processed and presented on the tumor cell surface because of the presence of GAr domain within EBNA1 (8, 9). We further showed that, consistent with this notion, the $CD4^+$ T cell response was responsible for T cell–mediated inhibition of BL growth in vivo (Figure 7C). These results suggest that $CD4^+$ T cells, after activation by EBNA1-$P_{607-619}$ peptide, play an important role in the inhibition of BL tumor growth in vivo. More importantly, the BL grew progressively in EBNA1-$P_{607-619}$–immunized CD4 KO mice but were significantly inhibited in the immunized CD8 KO mice (Figure 8A). Experiments with MHC class I KO and class II KO mice further confirmed the role of EBNA1-specific $CD4^+$ T cells in antitumor immunity (Figure 8B). Taken together, these findings suggest that the induction of EBNA1-$P_{607-619}$ peptide–specific $CD4^+$ T cells by DC/peptide vaccination leads to significant inhibition of B6-BL/EBNA1 tumor growth in vivo. The new findings represent what we believe to be the first direct evidence that $CD4^+$ T cells are primarily responsible for the rejection of BL tumor expressing EBNA1 in vivo.

Although we recently demonstrated that HLA-B8–restricted EBNA1-specific $CD8^+$ T cells could be elicited from human PBMCs after multiple peptide stimulation (12), $CD4^+$ T cell response against EBNA1 is dominant (15, 17–19). Thus, $CD4^+$ T cell response in our tumor model resembles that in patients with EBV-associated tumor. Several mechanisms of $CD4^+$ T cell–mediated antitumor immunity have been proposed. Studies of EBNA1-specific $CD4^+$ T cell lines established from healthy human donors have shown that some $CD4^+$ Th1 cells can directly kill BL cells in an 18-hour ^{51}Cr release assay (18), which suggests that $CD4^+$ T cells might inhibit EBV-infected cells through cytotoxicity mediated by perforin or Fas ligand expressed by $CD4^+$ effector cells. Both perforin- and Fas-mediated cytotoxicity have been implicated in the clearance of murine gammaherpesvirus-68 (MHV-68), which has been used as a model of human EBV infection (23, 24). However, our EBNA1-$P_{607-619}$–activated T cells did not show any cytotoxic activity against B6-BL/EBNA1-GFP cells in either a 4-hour or a 16-hour ^{51}Cr release assay. Alternatively, $CD4^+$ T cells might indirectly kill target cells through the production of cytokines, such as IFN-γ, which have been shown to have inhibitory activity in EBV-induced B cell growth (25, 26). The control of tumor growth by IFN-γ in other animal models, including models for MHC class II–negative tumors (27, 28), is well established (29–31), although conflicting results have also been reported (32–34). Inhibition of angiogenesis rather than direct arrest of tumor cell proliferation has been attributed to IFN-γ–mediated antitumor immunity (35). We are currently investigating these possibilities using various types of KO mice.

It has been suggested that immunocompromised individuals such as HIV-infected patients have increased risk of developing BL, which is strongly associated with EBV (36–38). These studies suggest that the host immune system plays an important role in con-

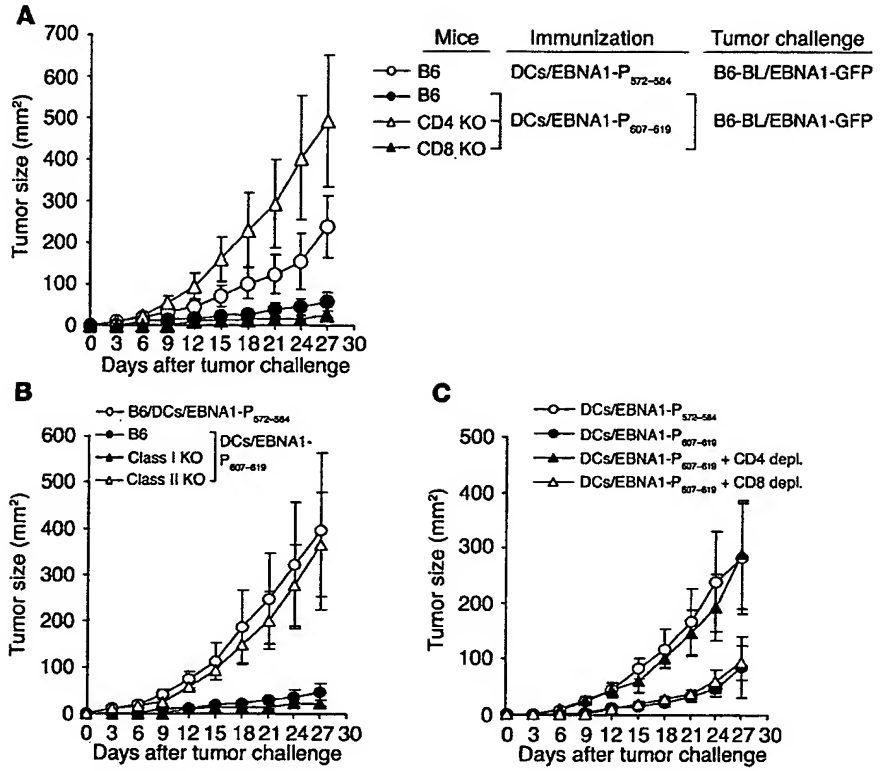

Figure 8

CD4⁺ T cells are responsible for the inhibition of BL cells in vivo. (**A**) Determination of T cell subsets responsible for the observed antitumor immunity. Wild-type, CD4 KO, and CD8 KO mice were immunized with DCs/EBNA1-$P_{607-619}$ peptide, and 2 weeks later were challenged with 5×10^5 B6-BL/EBNA1-GFP tumor cells. The tumor sizes were measured every 2 days after tumor challenge. Significant inhibition of tumor growth was observed in wild-type and CD8 KO mice immunized with DCs/EBNA1-$P_{572-584}$ peptide compared with other groups ($P = 0.005$), Similar results were obtained in 3 repeated experiments. (**B**) Antitumor immunity elicited in B6, MHC class I KO, but not in MHC class II KO mice. B6, class I, and class II KO mice were immunized with DCs/EBNA1-$P_{607-619}$ and were then challenged with B6-BL/EBNA1-GFP cells. DC/EBNA1-$P_{572-584}$ served as a specificity control. Significant suppression of tumor growth was observed in B6 and MHC class I KO mice immunized with DC/EBNA1-$P_{607-619}$ peptide compared with other groups ($P = 0.0065$). (**C**) Depletion (depl.) of the subset of CD4⁺ T cells abolished their ability to suppress tumor growth. The immunized mice were treated with anti-CD4 (GK1.5) or anti-CD8 (2.43) mAb's (200 μg in 500 μl volume) 1 day before tumor challenge and on days 1, 3, and 5 after challenge. Tumor growth was not inhibited in mice with depletion of CD4⁺ T cells, while depletion of CD8⁺ T cells did not affect antitumor immunity ($P = 0.0127$). B6 mice immunized with DC/EBNA1-$P_{607-619}$ or DC/EBNA1-$P_{572-584}$ peptide served as positive and negative controls, respectively.

trolling the development of BL. However, the relationship between immunocompromise and EBV-associated cancer is still poorly understood. In particular, it is not known whether the number and function of CD4 T cells are correlated with the development of EBV-associated BL. Since our results suggest that the induction of EBNA1-specific CD4⁺ T cells is critical in controlling the growth of EBNA1-expressing BL tumor model in vivo, it is important to identify MHC class II–restricted EBNA1 epitopes recognized by CD4⁺ T cells. Such epitopes could be used to stimulate CD4⁺ T cells specific for EBNA1 and then adoptively transferred along with EBV-specific CD8⁺ T cells into patients with EBV-associated cancer. Alternatively, these MHC class II–restricted EBNA1 peptides could be used in a vaccine in combination with MHC class I viral peptides to elicit both CD4⁺ and CD8⁺ T cell responses. It has been demonstrated that both CD4⁺ and CD8⁺ T cell responses are required for controlling the outgrowth of EBV-transformed B cells in seropositive donors (39). Thus, it is critical to include both MHC class I– and class II–restricted peptides from EBV antigens in cancer vaccines for recruiting and activating CD4⁺ and CD8⁺ T cell responses in clinical setting, ultimately leading to tumor destruction.

Methods

Mice. C57BL/6 (B6, H-2^b) female mice were obtained from the National Cancer Institute. CD4 KO and CD8 KO mice in a B6 background were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and had been backcrossed for more than 10 generations. MHC class I– and class II–deficient ($\beta 2m^{-/-}$ and I-$A^{b-/-}$, respectively) mice that had been backcrossed to the B6 background for more than 12 generations were purchased from Taconic (Germantown, New York, USA). All mice were maintained in the animal facility at Baylor College of Medicine under specific pathogen-free conditions and were used at 8–12 weeks of age. All studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine regarding the use of laboratory animals.

Cell lines. A murine BL cell line, designated B6-BL, was initially derived from human *Igλ-MYC* transgenic mice in a C57BL/6 background (20). The B6-BL/EBNA1 cell line was derived from *Igλ-MYC* × EBNA1 double-transgenic mice. Both B6-BL and B6-BL/EBNA1 cell lines were kindly provided by Herbert. C. Morse III and Ted Torrey at the National Institute of Allergy and Infectious Diseases, NIH. EBNA1 transgenic mice were independently generated using the method and constructs similar to one previously described (ref. 40; Ted Torrey, personal communication), and were used to cross with the *Igλ-MYC* transgenic mice. However, the EBNA1 expression in B6-BL/EBNA1 cell line could be detected by RT-PCR but not by Western blot analysis. Therefore, we introduced EBNA1 into B6-BL cells by a retroviral vector encoding the *EBNA1-GFP* gene. Retroviral EBNA1 constructs and viral supernatant preparation were conducted as previously described (12, 41). Expression of EBNA1-GFP was under the control of viral long-terminal repeat promoter. The resultant cell line was designated B6-BL/EBNA1-GFP. As a control, we generated a B6-BL/GFP cell line. We also generated an additional tumor cell line, RM1/NY-ESO-1-GFP, by introducing the *NY-ESO-1-GFP* fusion gene into the murine RM1 prostate cell line. The human embryo kidney 293 cell line expressing mouse MHC class II (I-A^b) molecules was previously described (42). These cell lines were maintained in RPMI 1640 medium (GIBCO; Invitrogen Corp., Carlsbad, California, USA) supplemented with 10% FBS (Gemini Bio-Products, Woodland, California, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

EBNA1 peptides. The peptides were synthesized by a solid-phase method using a peptide synthesizer (model AMS 422; Gilson Co., Worthington, Ohio, USA) and were purified by HPLC and were more than 98% pure. The mass of some peptides was confirmed by mass spectrometry analysis. EBNA1 peptide sequences were identical to those previously described (19).

DC preparation and immunization. Bone marrow–derived DCs from C57BL/6 mice were prepared as previously described (42). In some experiments, B6 mice were immunized with 50 μg of full-length EBNA1 (19) or GAr-del-EBNA1 protein (a kind gift of Jindong Wang, University of Wisconsin, Madison, Wisconsin, USA) or 100 μg of EBNA1 peptides emulsified in an equal volume of CFA (Sigma-Aldrich, St. Louis, Missouri, USA) in a total volume of 50 μl.

T cell stimulation, cytokine release, and ELISPOT assay. Two million splenocytes were freshly prepared from the immunized mice (2 per group) and incubated with various EBNA1 peptides at a final concentration of 10 μM in RPMI 1640/5% mouse serum (Valley Biomedical Inc., Winchester, Virginia, USA) and cultured in a 24-well plate (Corning, Corning, New York, USA) at 37°C in 5% CO_2 for 6 days. These T cells were tested for their ability to recognize several tumor target cells or peptide-pulsed targets. In some experiments, T cells from splenocytes or draining lymph node cells of the immunized mice were directly tested for their ability to recognize target cells. Murine IFN-γ release was determined with ELISA kits (Endogen Inc., Woburn, Massachusetts, USA) according to the manufacturer's instructions. T cell activities against tumor target cells were also determined by ELISPOT, as previously described (43).

Intracellualr cytokine staining and flow cytometric analysis. Spleen cell cultures stimulated with EBNA1 peptides for 18 hours were established as described above. Cytokine secretion from T cells was then blocked by the addition of brefeldin A (10 μg/ml; Sigma-Aldrich) for 3 hours before harvesting. Cells were washed once in FACS buffer (1% FCS-PBS) and adjusted to 0.5×10^6/tube and stained for expression of CD4 and CD8 by phycoerythrin-conjugated (PE-conjugated) anti-CD4 (GK1.5) and anti-CD8 (53-6.7), respectively. After 30 minutes on ice, the cells were washed twice, then fixed with 2% paraformaldehyde-PBS for 20 minutes at 4°C, followed by intracellular staining in permeabilization buffer containing 0.5% saponin and 1% BSA in PBS. After incubation with 1 μg/tube FITC-conjugated anti-IFN-γ (XMG1.2; BD Biosciences – Pharmingen, San Diego, California, USA) for 45 minutes at 4°C. and the cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry.

B6-BL, B6-BL/GFP, and B6-BL/EBNA1-GFP tumor cells were washed once in FACS buffer, adjusted to 0.2×10^5/tube, and stained for cell surface markers by incubation with 1–2 μg/tube of PE-conjugated anti-mouse mAb's (all from BD Biosciences – Pharmingen): anti-B220 (RA3-6B2), anti-H-2K^b (AF6-88.5), anti-H-2I-A^b (AF6-120.1), anti-B7.1 (16-10A1), anti-B7.2 (GL1), and anti-ICAM-1 (3E2). After 30 minutes on ice, cells were washed twice with FACS buffer and analyzed with a FACScan flow cytometer (BD, San Jose, California, USA).

MTT assay. Cells were seeded in a flat-bottomed, 96-well plate at 2×10^4 cells/well in RPMI-1640 plus 10% FCS. Before harvesting, 50 μl of the vital dye MTT (Sigma-Aldrich) in PBS (5 mg/ml) was added to the cultures. The blue dye taken up by the cells after 4 hours of incubation was dissolved in DMSO (100 μl/well). Readouts were taken at a 550 nm wavelength using an automated microplate reader.

Animal study. B6 mice and mice deficient in CD4, CD8, or MHC class I or class II molecules were immunized with EBNA1 peptides pulsed on B6 DCs (3×10^5/mouse) through tail veins. Two weeks later, mice were challenged with various tumor cells by subcutaneous injection of 5×10^5 of tumor cells. In some experiments, CD4$^+$ and CD8$^+$ T cells were depleted by intraperitoneal injection of 500 μl (containing 200 μg) of anti-CD4 (GK1.5) and anti-CD8 (2.43) mAb's, respectively, as previously described (42). Tumor growth was measured with a caliper every 2–3 days and the results described as tumor area in mm^2. Statistical significance was calculated with the two-sided Student's *t* test.

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Address correspondence to: Rong-Fu Wang, The Center for Cell and Gene Therapy, Baylor College of Medicine, ALKEK Building, N1120, One Baylor Plaza, Houston, Texas 77030, USA. Phone: (713) 798-1244; Fax: (713) 798-1263; E-mail: rongfuw@bcm.tmc.edu.

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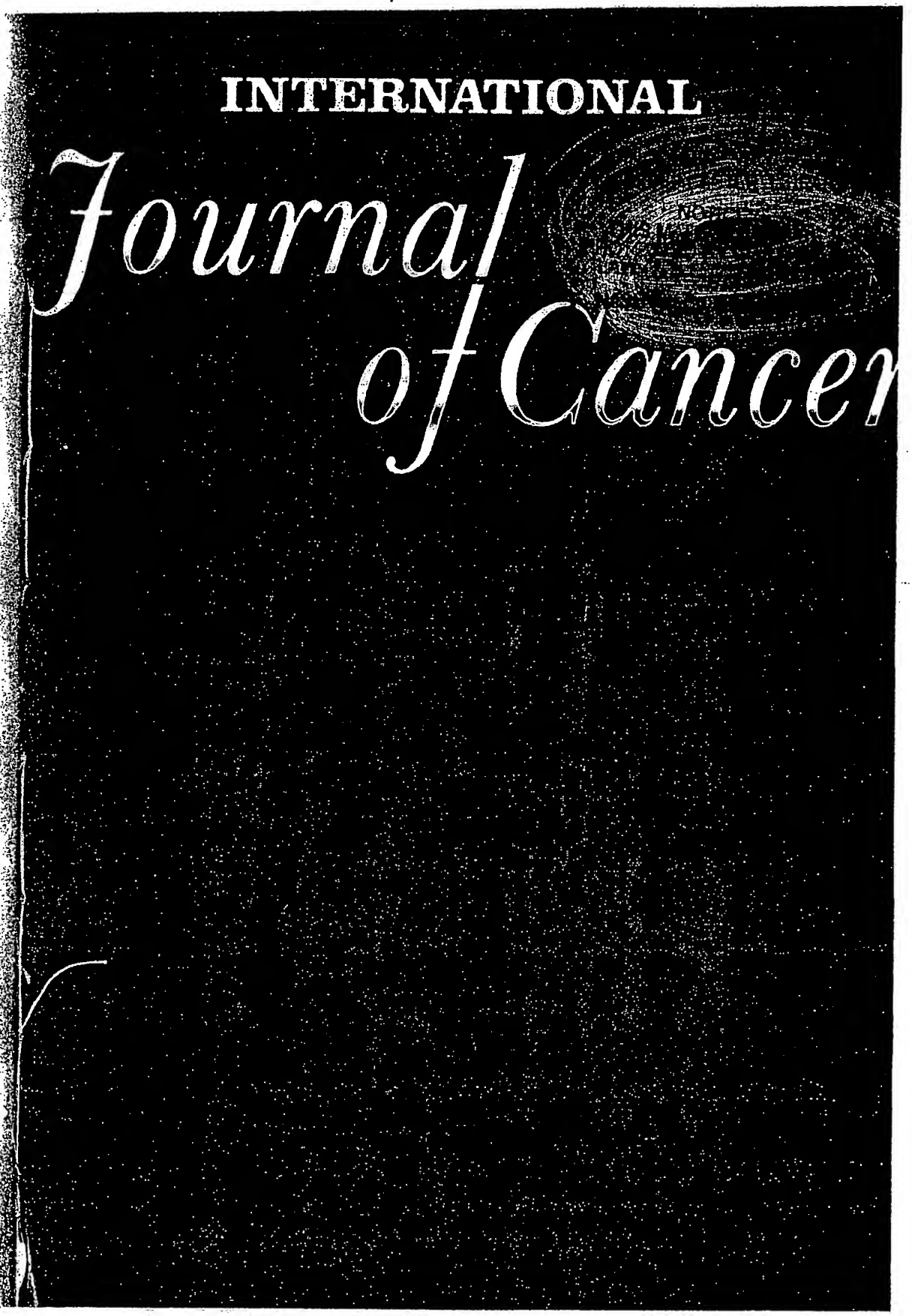
Exhibit 2



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Int. J. Cancer: 11, 499-520 (1973)

CELLULAR LOCALIZATION OF AN EPSTEIN-BARR VIRUS (EBV)-ASSOCIATED COMPLEMENT-FIXING ANTIGEN IN PRODUCER AND NON-PRODUCER LYMPHOBLASTOID CELL LINES

by

Beverley M. REEDMAN [1] and George KLEIN

Department of Tumor Biology, Karolinska Institutet, S 104 01 Stockholm 60, Sweden

Anti-complement immunofluorescence (ACIF) was used to study the complement-fixing antigens of human lymphoblastoid cell lines. These cell lines carry the Epstein-Barr virus (EBV) genome although only producer cultures synthetize EBV-specific antigens (virus capsid antigen, VCA and early antigen, EA) detectable by direct and indirect immunofluorescence, usually in less than 5% of the cells. The ACIF test revealed an antigen localized in the nucleus of the lymphoblastoid cells. In contrast to EA and VCA, this antigen was present in over 90% of the cells of both producer and non-producer cultures. The antigen was shown to be specific for EBV by comparing the reactions of 52 sera in the ACIF test. Sera giving the nuclear reaction contained antibodies to VCA, EA or antigens detectable by complement fixation tests on cell extracts, but sera without EBV antibodies failed to give the reaction. Weak, equivocal or discordant reactions occurred with six sera with low titres in VCA, EA or complement fixation tests. Cell lines derived by transformation of human and primate lymphocytes by EBV gave the nuclear reaction. Control cells with no known association with EBV were non-reactive. These included foetal lymphocytes transformed by phytohaemagglutinin, cell lines derived from breast cancer, glioma, normal glia, pleuritis maligna and myeloma, and two marmoset lymphoid lines carrying Herpesvirus saimiri (HVS). In preliminary experiments, the ACIF test was used as a tool to trace the EBV genome at the cellular level. Cells from two Burkitt lymphoma biopsies, one tested after biopsy and one after passaging in nude mice, contained an EBV-specific antigen. Three clones of cells derived from hybrids of mouse somatic cells and a human lymphoblastoid cell line also contained such an antigen, but the number of reactive cells varied from clone to clone. A fourth clone was non-reactive.

A non-permissive interaction between EBV and human lymphoid cells is believed to be essential for the unlimited proliferation of human lymphoblastoid cell lines *in vitro*. Cord blood cells and foetal lymphocytes do not spontaneously develop into cell lines until and unless EBV is added (Pope *et al.*, 1968; Chang *et al.*, 1971; Nilsson *et al.*, 1971). As far as they have been tested, all lymphoblastoid cell lines of human origin contain complement-fixing antigens specific for EBV (Pope *et al.*, 1969; Vonka *et al.*, 1970*b*). DNA/DNA and cRNA/DNA hybridization showed that these lines also carry the EBV genome (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972). In contrast, virus-associated antigens detectable in fixed cells by direct and indirect immunofluorescence tests occur, by definition,

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[1] Recipient of D.B. Duncan Training Fellowship from the Queensland Cancer Fund; on leave from the Queensland Institute of Medical Research. Present address: c/o Queensland Institute of Medical Research, Herston Road, Herston, Queensland, Australia 4006.

only in producer cell lines. These antigens, EA and VCA, are normally detectable in less than 5% of the cells of the culture. VCA-producing cells were shown to be in various stages of degeneration (Henle and Henle, 1966*a*, *b*), and host-cell DNA, RNA and protein synthesis was considerably inhibited in EA-producing cells of producer cultures and non-producer cultures superinfected with EBV (Gergely *et al.*, 1971*a*, *b*). It is probable, therefore, that EA synthesis signals the entry of a cell into a lytic though often abortive virus cycle, and both EA and VCA synthesis are indicative of cell death.

The T antigens induced by the oncogenic papovaviruses and adenoviruses are virus-specific antigens compatible with continued cell life and multiplication in contrast to the V antigens which are exclusively limited to the lytic virus cycle. One purpose of the present study was to determine whether the human lymphoblastoid cell lines synthetize antigens analogous to the T antigens. It has been suggested that the EBV-specific complement-fixing antigens in producer and non-producer cell lines may represent such an antigen (Armstrong *et al.*, 1966; Pope *et al.*, 1969; Vonka *et al.*, 1970*a*, *b*) but direct and indirect immunofluorescence tests such as are currently used to detect EA and VCA have failed to demonstrate this antigen at the cellular level. It is therefore not known whether the complement-fixing antigen is a product of each cell in a culture, or is limited to a very small minority of cells diverted into an abortive virus cycle, thereby excluding themselves from the continued growth of the line. Since ACIF is reported to be more sensitive than direct or indirect immunofluorescence (Goldwasser and Shepard, 1958; Hinuma and Hummeler, 1961; Hinuma *et al.*, 1962) it was used here to study EBV-related complement-fixing antigens.

MATERIAL AND METHODS

Cell lines

The cell lines studied will be described with the results. Human lymphoblastoid cell lines from various sources were propagated as stationary suspension cultures in RPMI 1640 with 15% foetal calf serum. B82 mouse fibroblasts, Daudi/A9 or HBT cells were grown as monolayers in Eagle's MEM, and Daudi/A9 P2, clone 10 and clone 9 in HAT medium. Other cultures were propagated in RPMI 1640. All cultures were fed twice weekly.

ACIF test

Smears were prepared in the cytocentrifuge, or by spreading a concentrated suspension of washed cells on clean slides, air-dried and fixed in chilled acetone. Human serum which gave no reaction with Raji cells in the ACIF test and which contained no detectable antibodies to EBV antigens by complement fixation or immunofluorescence, was used as a source of complement. All dilutions and washes were made in balanced salt solution (BSS: 0.8% NaCl, 0.014% $CaCl_2$, 0.04% KCl, 0.02% $MgSO_4.7H_2O$, 0.06% KH_2PO_4, 0.06% $Na_2HPO_4.2H_2O$, pH 6.9).

After fixation, the smears were dipped in BSS and treated with test serum (usually diluted 1/8 and inactivated at 56° C for 30 min) containing complement (final dilution 1/10) at 37° C for 30 min in a humid chamber. They were washed with stirring for 30 min, stained with a suitable dilution of FITC-conjugated anti-human β_{1C}/β_{1A} globulin (Hyland Laboratories, Los Angeles, California, USA) at 37° C for 30 min or 4° C for 90 min, washed again and mounted in BSS:glycerol 1:1. The slides were examined in a Leitz Ortholux microscope equipped with a vertical Ploem type illuminator at oil immersion (54× or 100×).

Rabbit antiserum specific for the β_{1C} component of guinea-pig complement was kindly provided by Dr. T. Tachibana, National Cancer Center, Tokyo. IgG prepared from this serum was conjugated with FITC and used in the ACIF test with guinea-pig serum as a source of complement.

Titration of sera

Sera were titrated for antibodies to VCA and EA (D and R components) (Henle and Henle, 1966*a*; Henle *et al.*, 1969; Henle *et al.*, 1971*a*, *b*). Complement fixation titres were determined against crude and soluble extracts of QIMR-WIL cells as previously described (Walters and Pope, 1971).

Two-colour immunofluorescence

P3HR1 smears were stained by ACIF as described above, then treated with TRITC-Iketumba conjugate at 4° C for 1 h. TRITC-Iketumba is a reference conjugate (Klein *et al.*, 1971) with antibodies to VCA and R components of the EA syst 1971*b*). Individual cells were exa fluorescence due to the FITC-ar 470 nm blue excitation light an escence due to the TRITC-546 nm green excitation light.

RESULTS

ACIF reactions of Raji, P3H human lymphoblastoid cell lines

The reaction of sera containing with non-producer Raji cells in resulted in the staining of the nu of the cells (Fig. 2). This fluoresce by first inactivating the compl for 30 min and was readily disti the weak cytoplasmic backgrou to complement and conjugate intensity of the reaction varied and even within a test from one ce was typically finely granular and nucleus. This is in contrast to th plasmic and nuclear staining EA-positive cells, rarely numbe 5%, as observed in direct and in fluorescence tests on producer ce staining morphologically similar t for Raji cells was found when El were tested with the producer lir LY-46. A small number of these also stained in the cytoplasm a complement and conjugate, in bo and absence of EBV-positive s brilliantly stained cells were ofter EA-positive sera were used. Thes studied by two-colour immuno described later.

Specificity of nuclear staining

In order to investigate the sp nuclear reaction, 52 sera wh characterized for EBV antibodi fluorescence and complement tested for reactivity with Raji, P3 cells in the ACIF test (Table I). C had detectable VCA and co antibodies and all gave nuclea serum, No. 38, was found to re with Raji cells but was either neg positive on repeated tests with

1971) with antibodies to VCA and both the D and R components of the EA system (Henle *et al.*, 1971*b*). Individual cells were examined for green fluorescence due to the FITC-anti β_{1C}/β_{1A} with 470 nm blue excitation light and for red fluorescence due to the TRITC-Iketumba with 546 nm green excitation light.

RESULTS

ACIF reactions of Raji, P3HR1 and LY-46 human lymphoblastoid cell lines

The reaction of sera containing EBV antibodies with non-producer Raji cells in the ACIF test resulted in the staining of the nuclei of over 90% of the cells (Fig. 2). This fluorescence was inhibited by first inactivating the complement at 56° C for 30 min and was readily distinguishable from the weak cytoplasmic background staining due to complement and conjugate (Fig. 3). The intensity of the reaction varied from test to test and even within a test from one cell to another but was typically finely granular and confined to the nucleus. This is in contrast to the brilliant cytoplasmic and nuclear staining of VCA- and EA-positive cells, rarely numbering more than 5%, as observed in direct and indirect immunofluorescence tests on producer cell lines. Nuclear staining morphologically similar to that described for Raji cells was found when EBV-positive sera were tested with the producer lines, P3HR1 and LY-46. A small number of these cells (up to 2%) also stained in the cytoplasm and nucleus with complement and conjugate, in both the presence and absence of EBV-positive sera. Additional brilliantly stained cells were often observed when EA-positive sera were used. These reactions were studied by two-colour immunofluorescence as described later.

Specificity of nuclear staining

In order to investigate the specificity of the nuclear reaction, 52 sera which had been characterized for EBV antibodies by immunofluorescence and complement fixation were tested for reactivity with Raji, P3HR1 or LY-46 cells in the ACIF test (Table I). Of these, 32 sera had detectable VCA and complement-fixing antibodies and all gave nuclear staining. One serum, No. 38, was found to react consistently with Raji cells but was either negative or weakly positive on repeated tests with P3HR1 cells. Fourteen sera were non-reactive in VCA, EA and complement fixation tests, and also failed to react in the ACIF test. The remaining six sera were also non-reactive in VCA and EA tests. Two of these (Nos. 19 and 20) had relatively high complement fixation titres and were reactive in the ACIF test, while another serum (No. 17) had a low level of complement-fixing antibodies and was non-reactive in the ACIF test. Two sera (Nos. 15 and 16) which were non-reactive in complement fixation were considered equivocal in the ACIF test because of the very weak reactions observed. Finally, one serum (No. 18) was recorded as positive in the ACIF test and may have had a low level of complement-fixing antibody. Thus there was a positive correlation between the presence of EBV-specific antibodies in the sera and the presence of antibodies giving the nuclear reaction in the ACIF test. Of the six sera which did not fit into this pattern two could be considered discordant in that they were VCA- and EA-negative but complement fixation and ACIF-positive; the other four sera gave borderline, equivocal or negative reactions in all these tests. The results are presented diagrammatically in Figure 1.

ACIF reactions of EBV-carrying and control cells

The reactivity of different types of cells in the ACIF test was investigated and the results are summarized in Tables III and IV. Smears of each cell type were tested with EBV-positive sera which reacted strongly with Raji cells and also with sera giving no reaction with Raji cells and having no detectable antibodies to EBV. Appropriate controls were included in each test.

All the human lymphoblastoid cell lines tested, regardless of origin and producer or non-producer status, reacted with EBV-positive sera in a manner similar to that described for Raji cells but failed to react with the negative sera (Table II, Figs. 4 and 5). This included five cell lines derived from transformation of cord-blood cells with EBV, as well as the virus donor line 883L.

Foetal liver cells, two preparations of cord-blood cells transformed by phytohaemagglutinin, Vero cells, mouse and human fibroblasts, and cell lines derived from glioma, breast cancer, normal glia, sarcoma and pleuritis maligna, all failed to give the Raji-type nuclear reaction in the ACIF test (Table III). Several atypical

TABLE I

REACTIONS OF EBV-POSITIVE AND -NEGATIVE SERA IN ACIF TESTS WITH LYMPHOBLASTOID CELL LINES

Serum No.	Code	Diagnosis [1]	VCA titre	EA titre [2]		Complement fixation titre		ACIF reaction [3]
				D	R	Crude	Soluble	
1	EK	control	<10	<10	<10	<8	<8	–
2	IE	control	<10	<10	<10	<8	<8	–
3	JH	control	<10	<10	<10	<8	<8	–
4	S481	control	<10	NT	NT	<8	<8	–
5	S504	control	<10	NT	NT	<8	<8	–
6	S559	control	<10	NT	NT	<8	<8	–
7	S564	control	<10	NT	NT	<8	<8	–
8	S578	control	<10	NT	NT	<8	<8	–
9	S584	control	<10	NT	NT	<8	<8	–
10	S659	control	<10	NT	NT	<8	<8	–
11	S874	control	<10	NT	NT	<8	<8	–
12	S881	control	<10	NT	NT	<8	<8	–
13	S883	control	<10	NT	NT	<8	<8	–
14	KCC1060	control	<10	NT	NT	<2	<2	–
15	S379	control	<10	NT	NT	<4	<4	±
16	S665	control	<10	<10		<4	<4	± [5]
17	RT.N.122	control	<10	<10		8	>4	–
18	KCC1036	control	<10	<10		⩽8 [4]	<8	+
19	DN	cpntrol	<10	<10		64	32	+
20	WL	control	<10	<10	<10	⩾256	⩾256	+
21	GD	control	10	<10	<10	16	16	+
22	KCC1437	Ca man.	10	<10	<10	16	16	+
23	IE	control	20	<10	<10	8	<8	+
24	AI (Q)	control	20	<10	<10	64/128	64	+
25	JC (Q)	control	40	<10	<10	64	64	+
26	LB (Q)	control	40	<10	<10	64	64	+
27	GI	control	40	<10	<10	16	16	+
28	MQ	control	40	<10	<10	32	16	+
29	BMA	control	40	<10	<10	⩾128	⩾128	+
30	(Q)	control	40	NT	NT	64	32	+
31	RH (Q)	control	40-80	<10	<10	128	128	+
32	TM	control	80	<10	<10	128	128	+
33	MG (Q)	control	80	<10	<10	128	>32	+
34	PG	control	80	<10	<10	32	16	+
35	ES	control	80	<10	<10	8	8	+
36	LG	control	80	<10	<10	64	64	+
37	KCC1433	NPC	80-160	20		32/64	32	+
38	BB	control	160	<10	<10	16	8	+ [6]
39	JM	control	160	<10	<10	32	16	+
40	S246	BL	160	NT		⩾128	64	+
41	KCC1444	NPC	160	<10	40	64	64	+
42	KCC1382	BL	320	<10		64	32	+
43	KCC1302	BL	320	10	160	64	32	+
44	S323	control	640	NT		64	64	+
45	2036	control	⩾640	NT		32-64	32	+
46	5027	control	⩾640	NT		⩾128	64	+
47	KCC1311	BL	640	10	160	128	128	+
48	KCC658	NPC	640	320		1024	⩾256	+
49	KCC875	BL	640-1250	640		256	⩾32	+
50	KCC848	BL	1280-2560	640		⩾256	⩾256	+
51	KCC812	BL	2560	320		512	⩾256	+
52	KCC1349	BL	2560	320	640	⩾256	⩾256	+

[1] BL = Burkitt's lymphoma, NPC = nasopharyngeal carcinoma, Ca man. = Cancer of mandible, NT = not tested.
[2] Where only one EA titre is indicated, no distinction was made between the D and R components.
[3] Sera were tested with Raji, P3HR1 or LY-46 cells, – = no nuclear reaction as described for Raji cells, ± = weak reaction, + = positive reaction.
[4] Serum anti-complementary at 1/8 dilution.
[5] Very weak reaction only observed after repeated testing.
[6] This serum was positive when tested with Raji cells, but negative or weakly positive with P3HR1 cells.

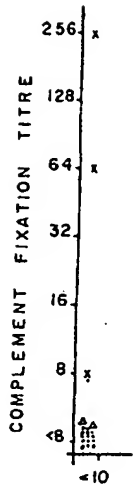


FIGURE 1
Relationsl
their reactio
● negative
reaction.

FIGURE 2
Positive reaction of Raji c
complement and FITC-anti β_{1C}

F TESTS

plement fixation titre		ACIF reaction [1]
rude	Soluble	
8	8	-
8	8	-
8	8	-
8	8	
8	8	
8	8	
8	8	
8	8	-
8	8	
8	8	
8	8	
8	8	
8	8	
2	2	
4	4	
4	4	
8	4	
8 [1]	8	+
64	32	+
256	256	+
16	16	+
16	16	+
8	8	+
1/128	64	+
64	64	+
64	64	+
16	16	+
32	16	+
128	128	+
64	32	+
128	128	+
128	128	+
128	32	+
32	16	+
8	8	+
64	64	+
2/64	32	+
16	8	+
32	16	+
128	64	+
64	64	+
64	32	+
64	32	+
64	64	+
2-64	32	+
128	64	+
128	128	+
1024	256	+
256	32	+
256	256	+
512	256	+
256	256	+

ndible, NT not tested.
ponents.
for Raji cells. ± weak reaction.

P3HR1 cells.

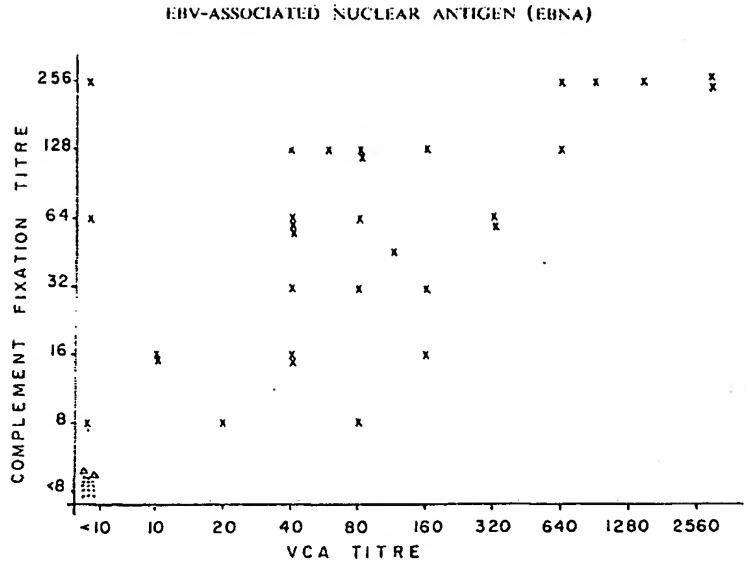


FIGURE 1

Relationship between VCA and complement-fixation titres of 52 sera and their reactions in the ACIF test with human lymphoblastoid cell lines. ● negative reaction; △ weak or equivocal reaction; × positive nuclear reaction.

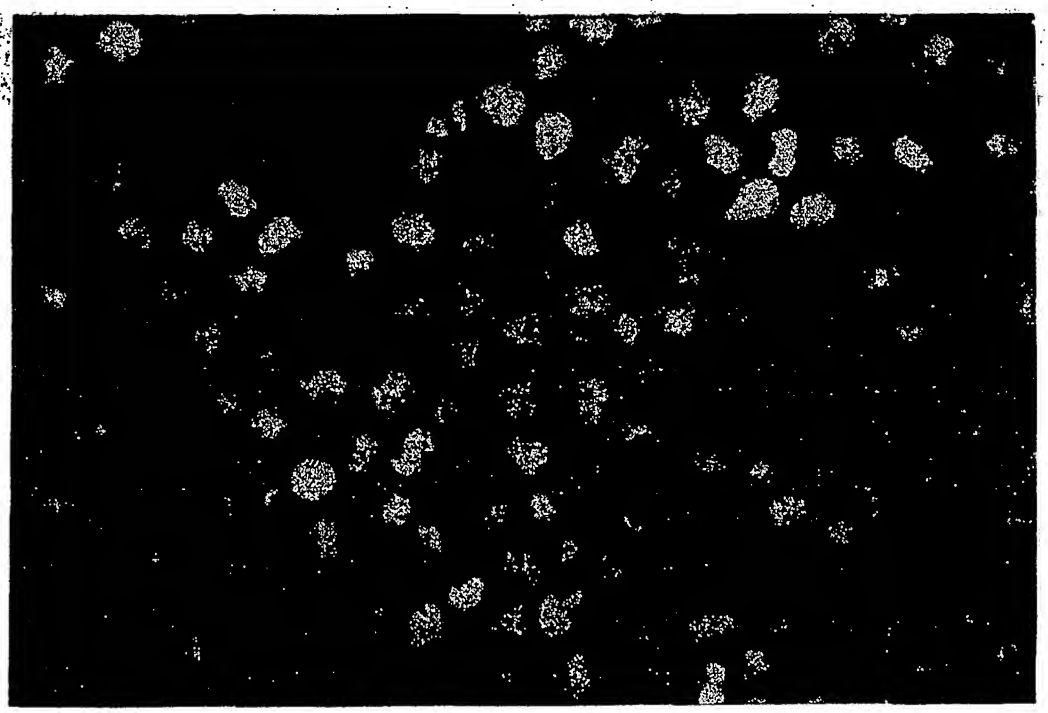


FIGURE 2

Positive reaction of Raji cells (human lymphoblastoid cell line, non-producer) with EBV-positive serum, complement and FITC-anti $\beta_1 C / \beta_{1A}$.

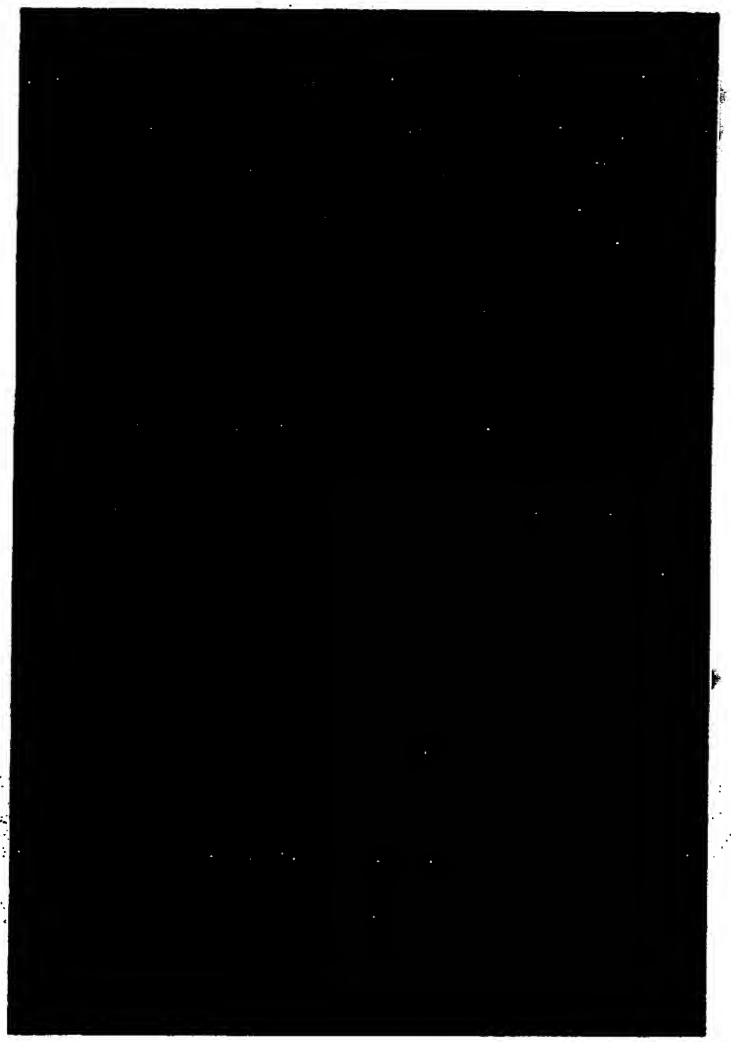


FIGURE 3

Negative reaction of Raji cells (human lymphoblastoid cell line, non-producer) with EBV-negative serum as complement source and FITC-anti β_{1C}/β_{1A}.

reactions were observed. These included the faint nuclear staining of the foetal liver cells by serum No. 42, and the spotty nuclear and cytoplasmic staining of the pleuritis maligna cells by sera 31, 38 and 49. The glia, sarcoma and myeloma cell lines contained a very low frequency of cells with stained nuclei.

Two squirrel monkey cell lines (KCSM 44 and KCSM 45) and one marmoset line (KCM 25), all of which grew as monolayers or as mixed cultures, were derived by transformation of peripheral white cells by EBV and were shown by EA and VCA tests to carry EBV. These three lines reacted in the ACIF test but another marmoset line (1670), which carried HVS but not EBV, was non-reactive (Table IV, Fig. 6). MLC, also a marmoset line carrying HVS, reacted weakly with one EBV-positive serum, No. 49, but not with four

TABLE II

REACTIVITY OF HUMAN LYMPHOBLASTOID CELL LINES WITH EBV-POSITIVE AND NEGATIVE SERA IN THE ACIF TEST

Cells	Origin [1]	EBV-[2] producer status	Detection of nuclear antigen in ACIF test [3]				Notes
			EBV-negative sera tested (All negative reactions)	EBV-positive sera tested		Conclusion [4]	
				±	+		
Akuba	BL	−	4 (1, 2, 3, 5)	4 (3)	2 (1, 2)	+	Nai-sto [5]
Daudi	BL	+	3 (1, 2, 3)	1 (4)	2 (1, 2)	+	Klein, E. *et al.*, 1968
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	-	[illegible]

nolayers or as mixed cul-
transformation of periph-
/ and were shown by EA
y EBV. These three lines
est but another marmoset
ied HVS but not EBV, was
Fig. 6). MLC, also a mar-
S, reacted weakly with one
lo. 49, but not with four

l line, non-
and FITC-

TABLE II

REACTIVITY OF HUMAN LYMPHOBLASTOID CELL LINES WITH EBV-POSITIVE AND NEGATIVE SERA IN THE ACIF TEST

Cells	Origin [1]	EBV- [2] producer status	Detection of nuclear antigen in ACIF test [3]				Notes
			EBV-negative sera tested (All negative reactions)	EBV-positive sera tested		Conclusion [4]	
				−	±	+	
Akuba	BL	−	4 (1, 2, 3, 5)	1 (3)	2 (1, 2)	+	Nai-sto [6]
Daudi	BL	+	3 (1, 2, 3)	1 (4)	2 (1, 2)	+	Klein, E. *et al.*, 1968
LY-46	BL	+	1 (1)		2 (2, 3)	+	Received from Dr. G. de Thé
Maku	BL	+	1 (6)		3 (2, 5, 6)	+	Yata and Klein, 1969
Namalva	BL	−	5 (1, 2, 3, 5, 6)	1 (3)	4 (1, 2, 5, 6)	+	Nai-sto [6]
P3HR1	BL	+	3 (1, 2, 6)		4 (1, 2, 5, 6)	+	Hinuma and Grace, 1967
Raji	BL	−	7 (1, 2, 3, 4, 5, 6, 7)		6 (1, 2, 3, 4, 5, 6)	+	Pulvertaft, 1965; Epstein *et al.*, 1966
LY-2	NPC	−	3 (1, 2, 5)	2 (2, 3)	2 (1, 4)	+	Received from Dr. G. de Thé
DSTC4	IM	−	3 (1, 2, 5)		4 (1, 2, 3, 4)	+	Junge *et al.*, 1971
JHTC33	IM	−	4 (1, 3, 5, 7)		3 (1, 2, 3)	+	Junge *et al.*, 1971
883L	IM	+	3 (1, 2, 3)		3 (1, 2, 3)	+	Miller *et al.*, 1971
SKL-1	Leukemia	−	3 (1, 2, 3)	2 (1, 3)	2 (2, 4)	+	Clarkson *et al.*, 1967
QIMR-WIL	Leukemia	+	2 (1, 2)		3 (2, 5, 6)	+	Pope, 1968
6410	Leukemia	−	4 (1, 2, 3, 5)	1 (3)	2 (1, 2)	+	Ikawata and Grace, 1964
T55A	Lymphosarcoma	NT [5]	4 (1, 2, 5, 6)	1 (1)	5 (2, 3, 4, 5, 6)	+	Dalton *et al.*, 1973
T36	Lymphosarcoma	NT	1 (6)		3 (2, 5, 6)	+	Dalton *et al.*, 1973
T57	Lymphoma	NT [5]	4 (1, 2, 5, 6)	1 (3)	5 (1, 3, 4, 5, 6)	+	Dalton *et al.*, 1973
T40	Melanoma	NT [5]	3 (1, 2, 5)		4 (1, 2, 3, 4)	+	Dalton *et al.*, 1973
T13	Paraganglioma	NT	2 (1, 2)		4 (1, 2, 5, 6)	+	Dalton *et al.*, 1973
F265	Normal	−	4 (1, 2, 3, 6)	1 (2)	2 (1, 3)	+	Durr *et al.*, 1970
NC37	Normal	+	1 (6)		3 (2, 5, 6)	+	Durr *et al.*, 1970
cb-8-1	TCB	−	2 (2, 6)	1 (5)	2 (1, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold
cb-8-2	TCB	−	2 (2, 6)	1 (2)	3 (1, 5, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold
cb-8-4	TCB	+	2 (2, 6)		4 (1, 2, 5, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold
cb-8-5	TCB	−	2 (2, 6)		4 (1, 2, 5, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold
cb-8-6	TCB	−	2 (2, 6)		4 (1, 2, 5, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold
cb-8-7	TCB	+	2 (2, 6)		4 (1, 2, 5, 6)	+	Received from Dr. J. Menezes and Dr. W. Leibold

[1] BL = Burkitt's lymphoma, NPC = nasopharyngeal carcinoma, IM = infectious mononucleosis, LS = lymphosarcoma, TCB = cord-blood cells transformed by a cell-free supernatant of 883L cells.

[2] Lines containing a significant frequency of EA or VCA-positive cells were classified as EBV producers (+); − = non-producer, NT = not tested.

[3] No. of sera tested; nos. in parenthesis = sera tested (see Table I) EBV-negative sera: 1 = No. 1, 2 = No. 3, 3 = No. 10, 4 = No. 12, 5 = No. 7, 6 = No. 2, 7 = No. 6.

[3] No. of sera tested; nos. in parenthesis = sera tested (see Table I) EBV-negative sera: 1 = No. 1, 2 = No. 3, 3 = No. 10, 4 = No. 12, 5 = No. 7, 6 = No. 2, 7 = No. 6. EBV-positive sera: 1 = No. 48, 2 = No. 32, 3 = No. 42, 4 = No. 50, 5 = No. 49, 6 = No. 31.

[4] − = negative reaction, ± = weak nuclear reaction, + = strongly positive.

[5] Cells reacting as described for Raji cells with several of the positive sera were regarded as positive (+). − = non-reactive.

[6] Nai-Sto = recently established line (unpublished). A biopsy was taken in Nairobi by Dr. S. Singh and flown to Stockholm where the line was established.

TABLE III

REACTIONS OF CONTROL CELLS AND BURKITT LYMPHOMA BIOPSIES IN THE ACIF TEST

Description of cells/cell lines	Detection of nuclear antigen in ACIF test [1]					Notes
	EBV-negative sera tested (all negative reactions)	EBV-positive sera tested [2]			Conclusion [3]	
		−	±	+		
Vero cell line: monolayer derived from green monkey kidney	2 (1, 6)	4 (1, 2, 5, 6)			−	
L cells: mouse fibroblast cell line	2 (1, 6)	3 (1, 2, 5)			−	Littlefield (1964)
Human lymphoid cells from foetal liver (biopsy)	4 (1, 3, 5, 7)	2 (1, 2)	1 (3) [4]		−	
Phytohaemagglutinin-transformed human cord-blood lymphocytes: preparation I	2 (5, 6)	4 (1, 2, 5, 6)			−	Received from Dr. W. Leibold
Phytohaemagglutinin-transformed human cord-blood lymphocytes: preparation II	2 (5, 6)	4 (1, 2, 5, 6)			−	Received from Dr. W. Leibold
HBT: monolayer derived from human breast cancer	2 (1, 6)	4 (1, 2, 5, 6)			−	Received from Dr. R. Bassin
E-622:						
monolayer derived from human pleuritis maligna	2 (5, 6)	4 (1, 2, 5, 6) [5]			−	Received from Dr. Marie-Rose Martin
monolayer derived from normal human glia	1 (6)	4 (2, 4, 6; 5-1‰) [6]			−	Received from Dr. Marie-Rose Martin
monolayer derived from human sarcoma	1 (6)	3 (2, 6; 4-3‰) [6]			−	Received from Dr. Marie-Rose Martin
monolayer derived from human glioma	1 (6)	3 (2-4‰; 5-38‰; 6-6‰) [6]	1 (4-220‰)		−	Received from Dr. Marie-Rose Martin
fibroblast culture derived from human foetal skin	1 (6)	2 (2, 5)			−	Received from Dr. H. Thé
RPMI No. 8226: suspension culture derived from human myeloma	1 (6)	3 (2-4‰; 5-4‰; 6-4‰) [6]				Received from Dr. J. Minowada
Burkitt lymphoma biopsy (Katana)	3 (1, 2, 3)	−	2 (1, 4)	2 (1, 2)	+ [7]	Biopsy taken in Nairobi by Dr. S. Singh and flown to Stockholm
Burkitt lymphoma biopsy (Margareth)	2 (1, 2)			2 (1, 2)	+	Received from Dr. J. Rygaard and Dr. C. O. Poulsen; tested after passaging nude mice (Poulsen *et al.*, 1973).

[1] No. of sera tested; nos. in parenthesis = actual sera tested as indicated in Table II.
[2] − = negative reaction, ± = weak nuclear reaction, + = strong nuclear reaction.
[3] Cell lines reacting as described for Raji cells with several of the positive sera were regarded as positive (+); − = negative.
[4] Faint lacy reaction only.
[5] Spotty reaction, unlike that described for Raji cells and not confined to the nucleus, was observed with positive sera 1, 5 and 6.
[6] ‰ of cells (= no. of cells par 1,000) with nuclear reaction with corresponding serum only is shown. If no figure is shown, serum was non-reactive.
[7] Coarsely granular reaction.

TABLE IV

COMPARISON OF EBV- AND HVS-TRANSFORMED PRIMATE LYMPHOCYTES BY THE ACIF TEST

Cell line	Origin	Culture	Transforming virus	Detection of nuclear antigen in ACIF test [1]					Notes
				EBV-negative sera tested (all negative reactions)	EBV-positive sera [2] tested			Conclusion [3]	
					−	±	+		
KCM 25	marmoset peripheral lymphocytes	Mixed monolayer and suspension	EBV	4 (1, 2, 3, 5)		1 (3)	2 (1, 2)	+	Received from Dr. L. Falk
KCSM 44	squirrel monkey	Mixed monolayer							

other positive sera. Several primate sera known to contain antibodies to HVS failed to give a nuclear reaction with MLC, 1670, KCSM 45 and Raji cells.

ACIF reactions of Burkitt lymphoma biopsies

Preparations of two Burkitt lymphoma biopsies were examined by ACIF (Table III). Cells from the first biopsy, Katana, were smeared and tested as for the cell lines. The nuclei of these cells reacted with EBV-positive sera (Fig. 7) but not with the EBV-negative sera (Fig. 8), but the fluorescence pattern was more coarsely granular than that described for Raji cells. The second tumor, Margareth, was tested after several passages in nude mice (Poulsen *et al.*, 1973). This was also reactive with a fluorescence pattern similar to that described for the lymphoblastoid cell lines.

ACIF reactions of Daudi/A9 hybrids

Four clones of cells derived by hybridization of mouse fibroblasts, A9, and a lymphoblastoid cell line, Daudi (Allerdice *et al.*, 1973), showed consistent differences in the number of antigen-positive cells (Table V, Fig. 9-12). The four clones are known to differ with regard to the number and type of human chromosomes they contain (Allerdice *et al.*, 1973). Present indications are that the reactivity of the hybrid clones can be correlated with the number of EBV-genome equivalents as determined by DNA hybridization (zur Hausen, personal communication).

Factors influencing the ACIF reaction

The reactions of the various cell lines, apart from the four clones just mentioned, have been described as positive or negative rather than as a percentage of positive cells. It was noted earlier that the intensity of the nuclear reaction varied from test to test and even within a test from one cell to another. It was found in practice that this variation made it difficult to quantitate the reaction. The quality of the smear seemed to be an important factor contributing to the success of the staining reaction. When Raji cells were smeared with a Pasteur pipette the intensity of the reaction varied from one section of the smear to another with perhaps only 50% of the cells brightly stained, but when the smears were prepared in the cytocentrifuge with the cells well separated from one another, over 90% of the cells usually stained intensely. Preparations

TABLE IV

COMPARISON OF EBV- AND HVS-TRANSFORMED PRIMATE LYMPHOCYTES BY THE ACIF TEST

Cell line	Origin	Culture	Transforming virus	Detection of nuclear antigen in ACIF test [1]				Notes
				EBV-negative sera tested (all negative reactions)	EBV-positive sera [2] tested		Conclusion [3]	
					−	±	+	
KCM 25	marmoset peripheral lymphocytes	Mixed monolayer and suspension	EBV	4 (1, 2, 3, 5)		1 (3)	2 (1, 2)	+ Received from Dr. L. Falk
KCSM 44	squirrel monkey peripheral lymphocytes	Mixed monolayer and suspension	EBV	2 (1, 2)			3 (1, 2, 3)	+ Received from Dr. L. Falk
KCSM 45	squirrel monkey peripheral lymphocytes	Mixed monolayer and suspension	EBV	4 (1, 2, 3, 5)		1 (3)	3 (1, 2, 5)	+ Received from Dr. L. Falk
MLC	marmoset lymphocytes	suspension	HVS	5 (1, 2, 4, 5, 6)	4 (1, 2, 4, 6)	1 (5)		− Rabson *et al.*, 1971
1670	marmoset lymphocytes	suspension	HVS	5 (1, 2, 4, 5, 6)	5 (1, 2, 4, 5, 6)			− Received from Dr. L. Falk

[1] No. of sera tested; nos. in parenthesis = actual sera tested as indicated in Table II.
[2] − = negative reaction, ± = weak nuclear reaction, + = strong nuclear reaction.
[3] Cell lines reacting as described for Raji cells with several of the positive sera were regarded as positive (+); − = negative.

Burkitt lymphoma biopsy (Margareth)

Dr. C. O. Poulsen; tested after passaging nude mice (Poulsen *et al.*, 1973).

[1] No. of sera tested; nos. in parenthesis = actual sera tested as indicated in Table II.
[2] − = negative reaction, ± = weak nuclear reaction, + = strong nuclear reaction.
[3] Cell lines reacting as described for Raji cells with several of the positive sera were regarded as positive (+); − = negative.
[4] Faint lacy reaction only.
[5] Spotty reaction, unlike that described for Raji cells and not confined to the nucleus, was observed with positive sera 1, 5 and 6.
[6] ‰ of cells (= no. of cells per 1,000) with nuclear reaction with corresponding serum only is shown. If no figure is shown, serum was non-reactive.
[7] Coarsely granular reaction.

FIGURE 4
Positive reaction of JHTC33 cells (human lymphoblastoid cell line, non-producer) with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}.

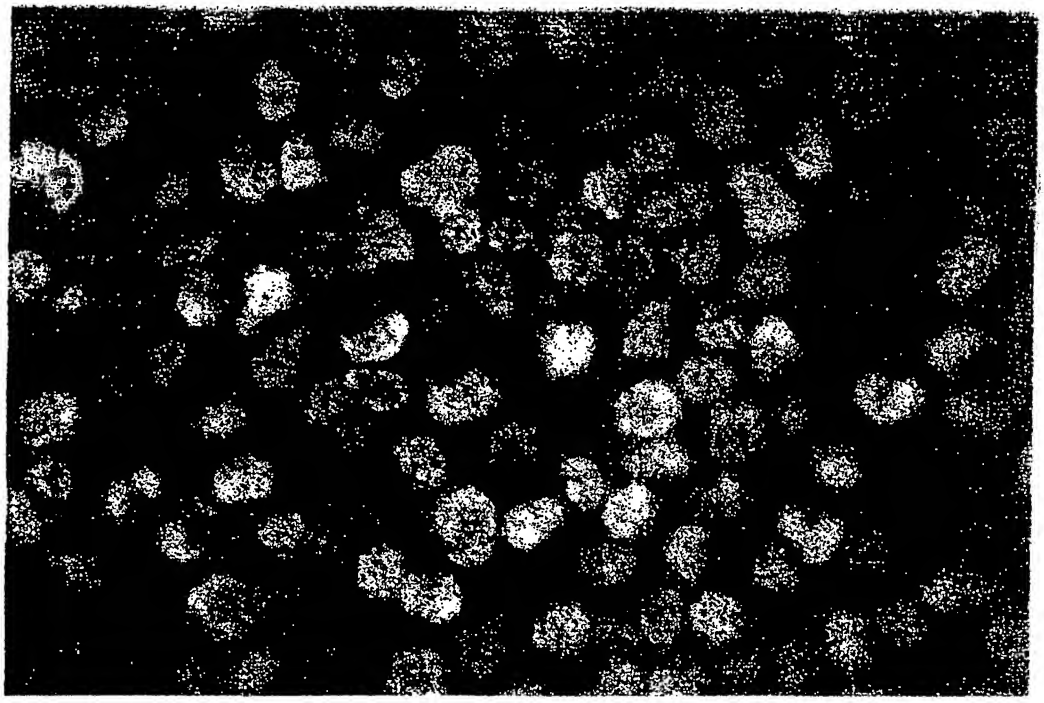
FIGURE 5
Positive reaction of 6410 cells (human lymphoblastoid cell line, non-producer) with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}.

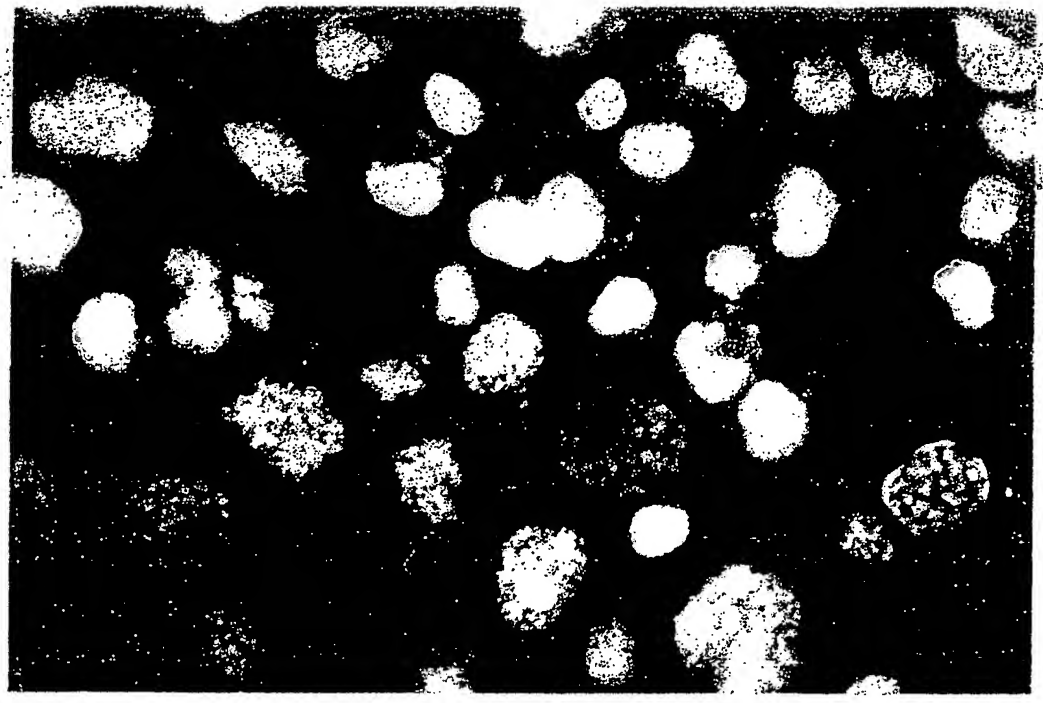
FIGURE 6
Negativ
EBV-negati

of the other lymphoblasto
tained very few negative
the reactions of the EBV
lines KCSM 44 and KCS
from cell to cell.

Recognition of EA-positive

The producer cell lines
which stained brilliantly i





of the other lymphoblasto
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lines KCSM 44 and KCS
from cell to cell.

Recognition of Epitopes

The producer cell lines
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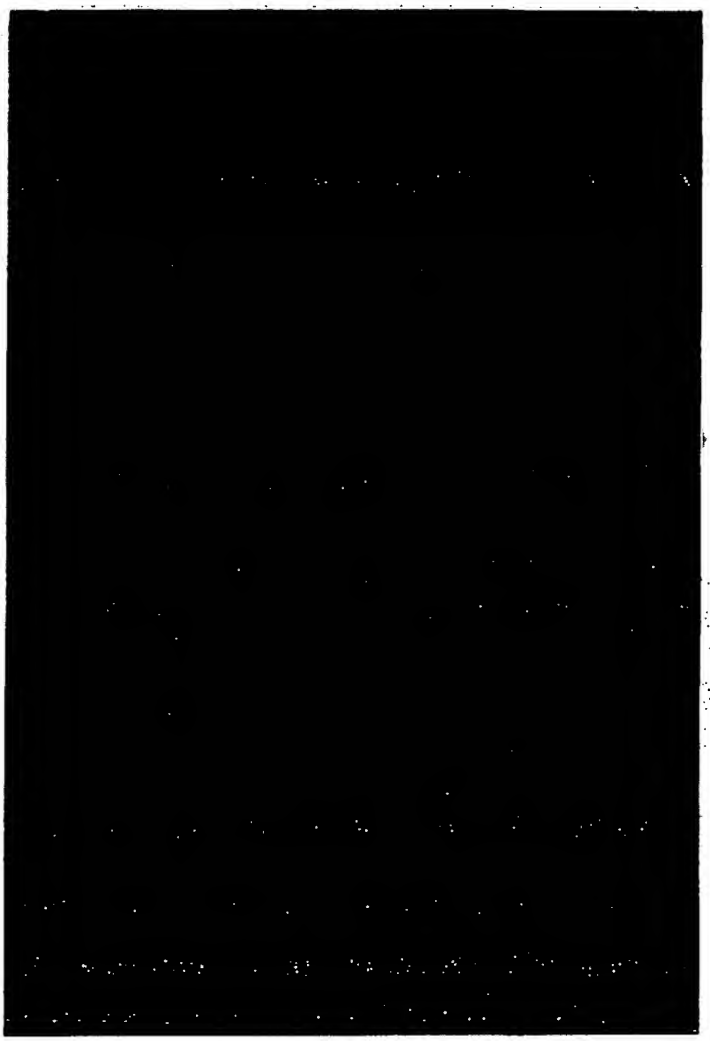
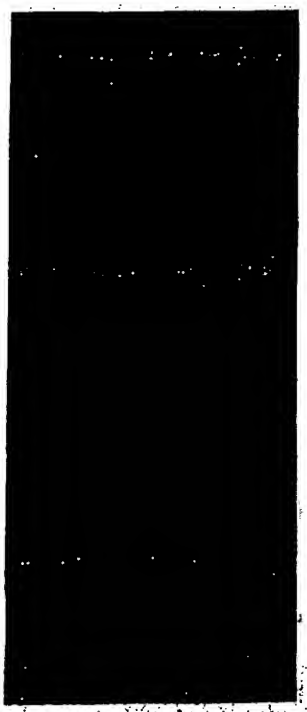
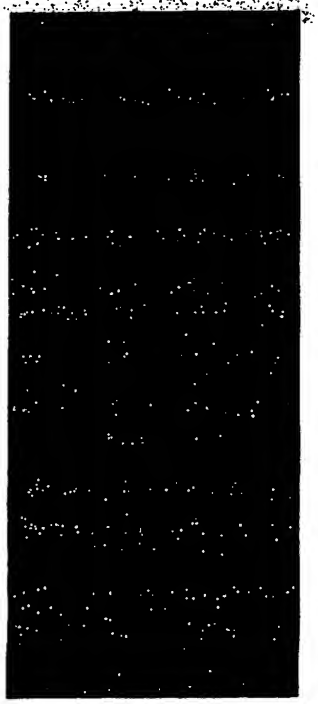


FIGURE 6

Negative reaction of 1670 cells (HVS-carrying marmoset cell line) with EBV-negative serum as complement source and FITC-anti β_{1C}/β_{1A}.



1-producer) with EBV-positive



lucer) with EBV-positive serum,

of the other lymphoblastoid cell lines also contained very few negative cells. The intensity of the reactions of the EBV-positive primate cell lines KCSM 44 and KCSM 45 varied markedly from cell to cell.

Recognition of EA-positive cells

The producer cell lines often contained cells which stained brilliantly in the cytoplasm and the nucleus with EA- and VCA-positive sera although this reaction was obscured if the nuclear staining was sufficiently bright. The reaction was most outstanding with EA-positive sera and cell lines with a high level of EA-producing cells. Two-colour immunofluorescence on P3HR1 cells showed that the EA-positive cells, *i.e.* those which stained red by direct TRITC-Iketumba rhodamine conjugate, were the same as those

FIGURE 7
Coarsely granular positive reaction of cells from a Burkitt lymphoma biopsy (Katana) with EBV-positive serum, complement and FITC-β_{1C}/β_{1A}.

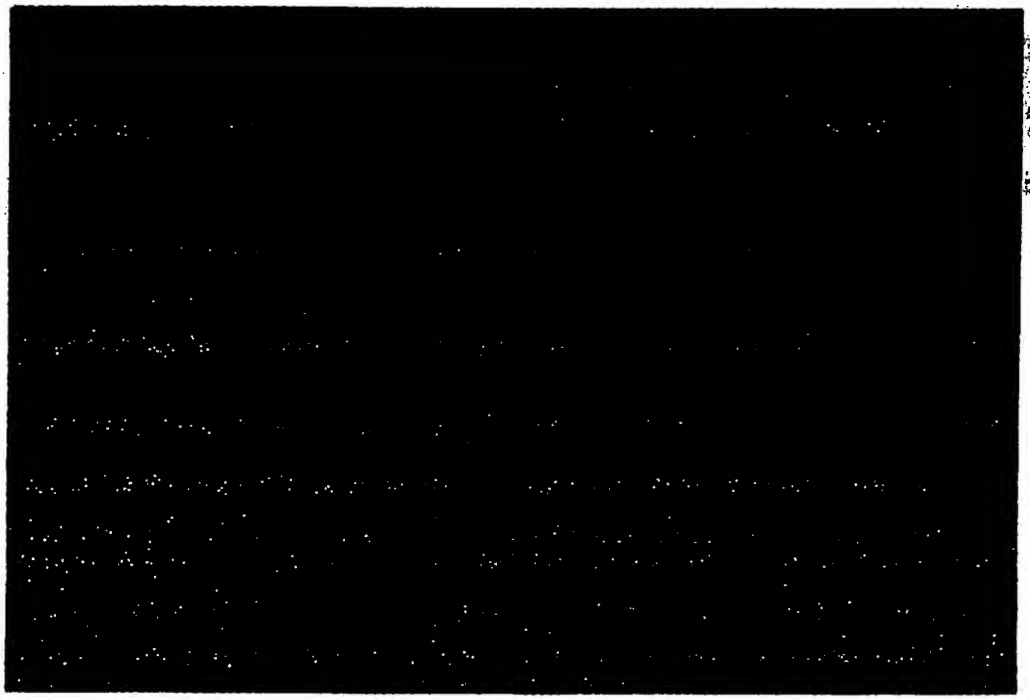


FIGURE 8
Negative reaction of cells from a Burkitt lymphoma biopsy (Katana) with complement, EBV-positive serum and FITC-anti β_{1C}/β_{1A}.

ACIF TESTS ON CL

Cell line
Daudi
Daudi/A9 OR
Daudi/A9-clone 10
Daudi/A9-clone 9
Daudi/A9-P2
L cells

[1] Percentage of cells with nu
EBV-negative sera.

FIGURE 9
Reaction of Daudi/A9
plement and FITC-anti β_{1C}

which stained brilliant g
serum in the ACIF test.
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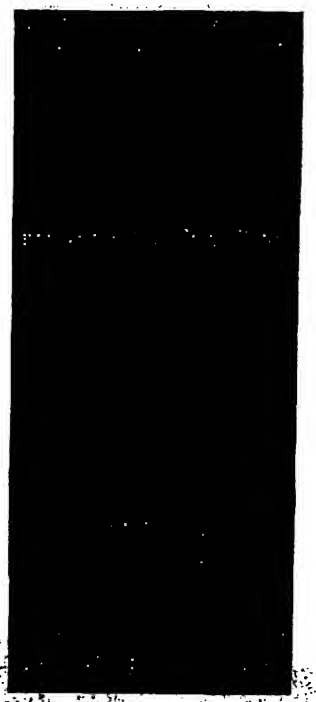
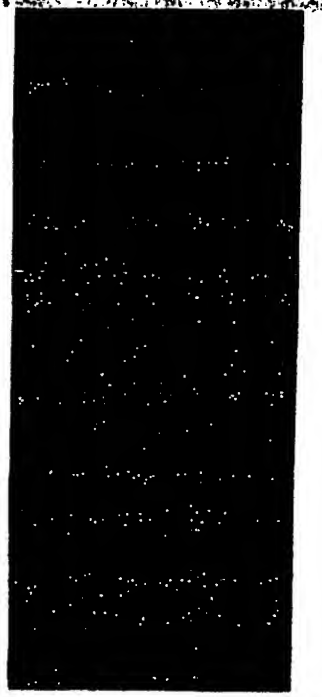
serum. These cells wer





sy (Katana) with EBV-positive

ith complement, EBV-positive

TABLE V

ACIF TESTS ON CLONES OF HYBRIDS OF A HUMAN LYMPHOBLASTOID CELL LINE (DAUDI) AND A MOUSE FIBROBLAST LINE (A9)

Cell line	% Reactive cells [1]	Origin of line
Daudi	>95	Klein, E. *et al.* (1968)
Daudi/A9 OR	58-90	Allerdice *et al.* (1973)
Daudi/A9-clone 10	29-34	" " "
Daudi/A9-clone 9	0.2-1.4	" " "
Daudi/A9-P2	0	" " "
L cells	0	Littlefield, 1964; A9 = mutant of mouse L cells

[1] Percentage of cells with nuclear fluorescence after reaction of smear with EBV-positive serum; the cell lines did not react with EBV-negative sera.

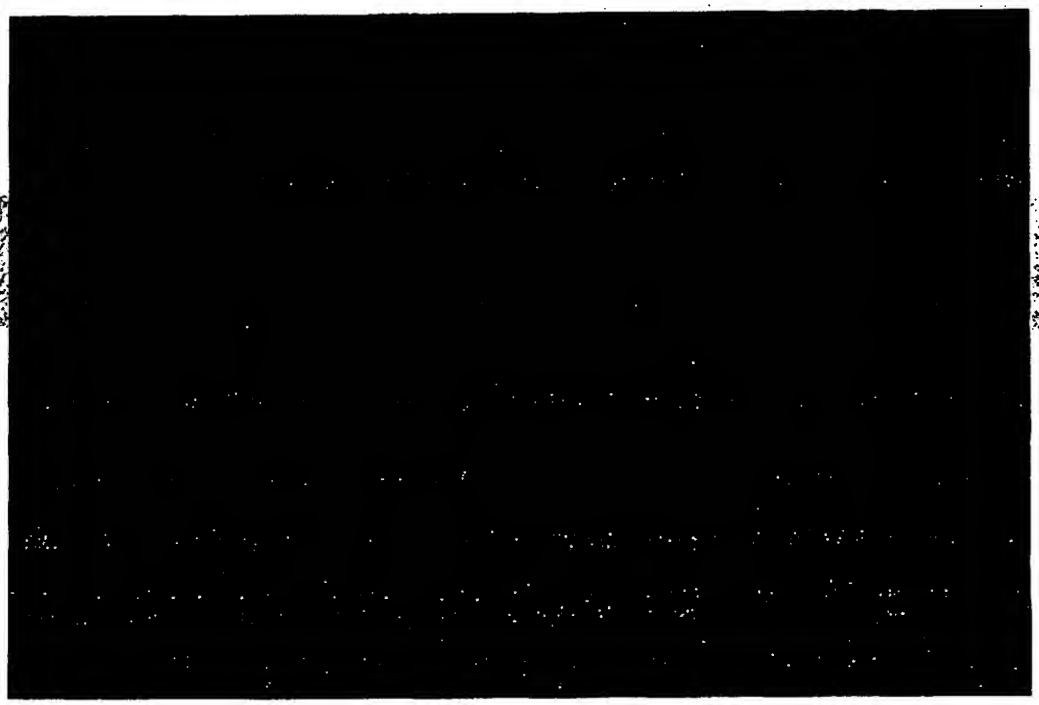


FIGURE 9

Reaction of Daudi/A9 cells clone OR (human-mouse hybrid clone) with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}. Approximately 80% of the cells gave nuclear staining.

which stained brilliant green by an EA-positive serum in the ACIF test. It was mentioned above that P3HR1 and LY-46 cell lines also contained some cells that reacted with complement and conjugate even in the absence of an EBV-positive serum. These cells were not identical to the EA-positive cells, however, when examined by two-colour immunofluorescence.

Association of nuclear antigen with chromosomes

Raji cells were hypotonized in 0.075 M KCl for 5 min before smears were prepared in the

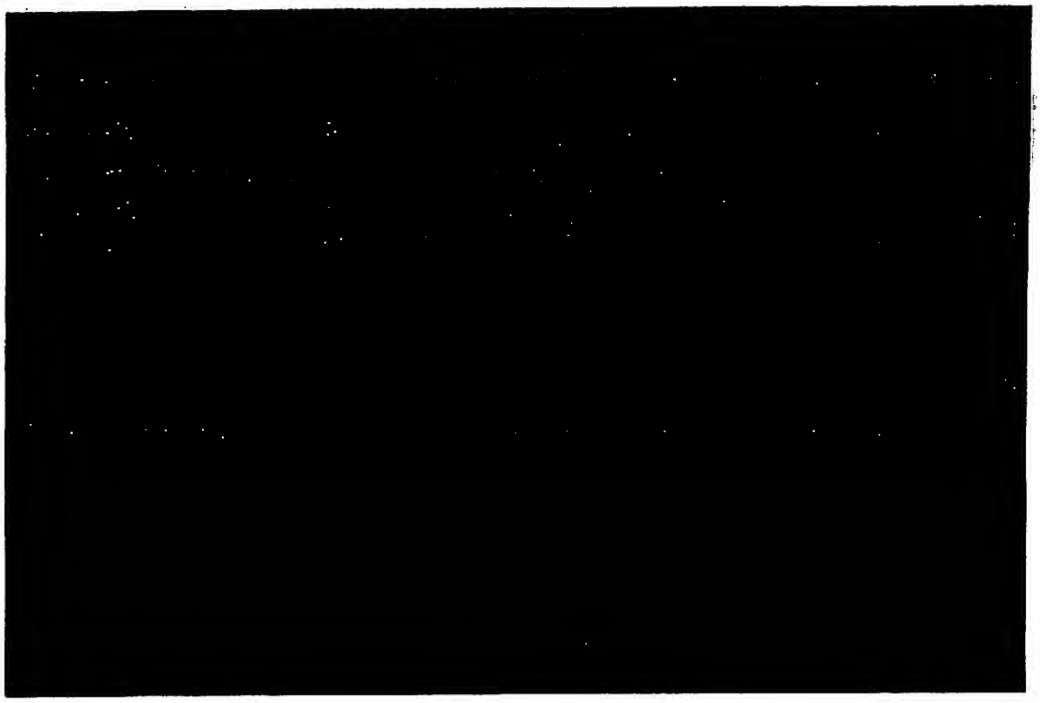


FIGURE 10

Reaction of Daudi/A9 clone 10 cells (human-mouse hybrid clone) with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}. Approximately 25% of the cells gave nuclear staining.

cytocentrifuge and subsequently stained by ACIF. The chromosomes in metaphase were clearly visible and stained by EBV-positive serum (Fig. 13). There was no fluorescence associated with the cytoplasm of the cell. Daudi/A9 clone OR cells were incubated with colchicine (0.04 μg/ml) for 4 h to arrest a large number of cells in metaphase. The cells were then smeared and stained and reactive cells in metaphase selected with the aid of phase contrast. Again, staining was associated with the chromosomes and not with the cytoplasm.

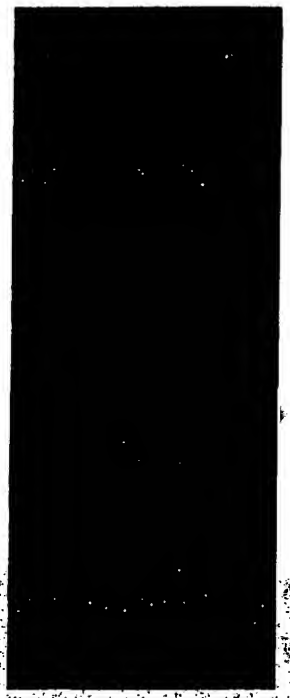
Differentiation of EBV-related and non-specific antinuclear antibodies

Several sera known to have antibodies to nuclear components of cells other than the EBV-carrying lymphoblastoid cells were compared with two of the EBV-positive sera from Table I for reactivity with the Raji cells and control cell lines (Table VI). The first EBV-positive serum (No. 32) reacted only with the Raji cells, while the second serum (No. 49) reacted strongly with the Raji cells, very weakly with the MLC cells, but not with the other, control cell lines. The other sera stained several or all of the control cell lines as well as the Raji cells. The non-specific fluorescence varied from coarsely granular to homogeneous, and in some cases was much brighter than the finely granular staining of the Raji cells (Fig. 14). The mouse fibroblasts seemed more refractory to staining than the other cell lines.

ACIF test with guinea-pig complement

Raji cells were examined by ACIF with guinea-pig serum as a source of complement instead of human serum. Guinea-pig complement and anti-guinea-pig β_{1C} conjugate stained the nuclei weakly at complement dilutions of 1/10 to 1/20 though not at hi 1/40). Human serum reacted with Raji cells at of 1/10 to 1/40 but onl was present with serum w The reaction with the nuclear reaction similar tc Raji cells with human se

FIGURE 11
Reactic
EBV-posit
1% of the



ı EBV-positive serum, containing.

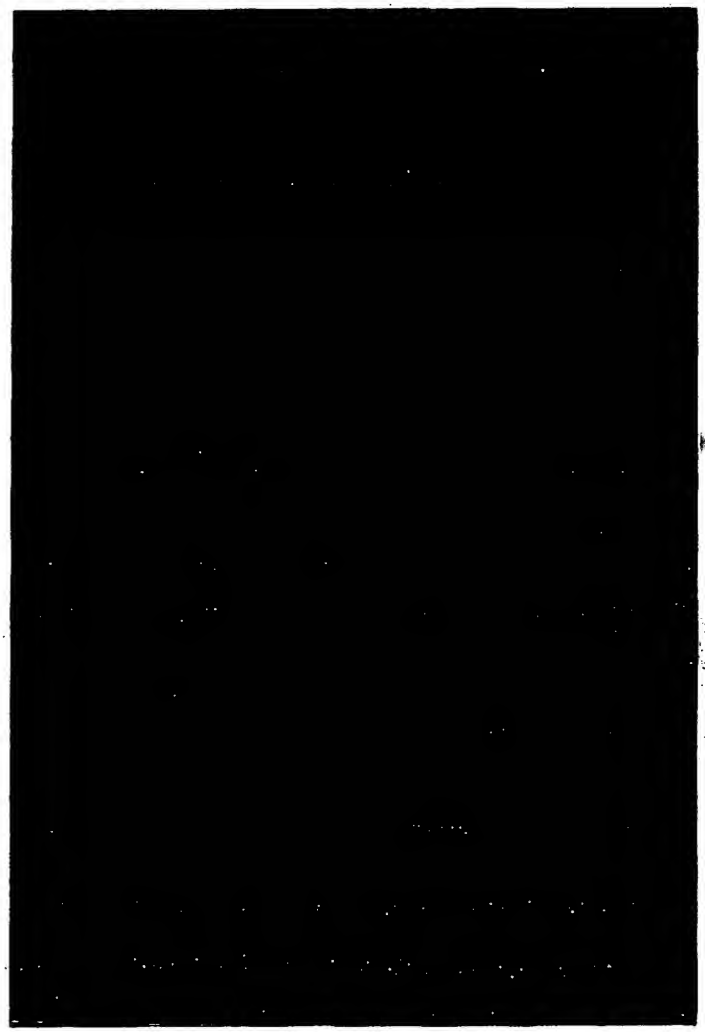


FIGURE 11

Reaction of Daudi/A9 clone 9 cells (human-mouse hybrid clone) with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}. Approximately 1% of the cells gave nuclear staining.

able VI). The first EBV-32) reacted only with the : second serum (No. 49) the Raji cells, very weakly but not with the other, : other sera stained several l cell lines as well as the ipecific fluorescence varied ır to homogeneous, and in h brighter than the finely he Raji cells (Fig. 14). The emed more refractory to r cell lines.

·pig complement

xamined by ACIF with a source of complement m. Guinea-pig complement β_{1C} conjugate stained the ıplement dilutions of 1/10 to 1/20 though not at higher dilutions (1/30 to 1/40). Human serum with EBV-antibodies reacted with Raji cells at complement dilutions of 1/10 to 1/40 but only background staining was present with serum without EBV antibodies. The reaction with the positive serum was a nuclear reaction similar to that described for the Raji cells with human serum as a complement source. It was easily distinguishable from the background due to the guinea-pig complement and conjugate, and was inhibited by first inactivating the complement at 56° C for 30 min.

DISCUSSION

Staining of the nuclei of cultured human lymphoblastoid cells by ACIF correlated with

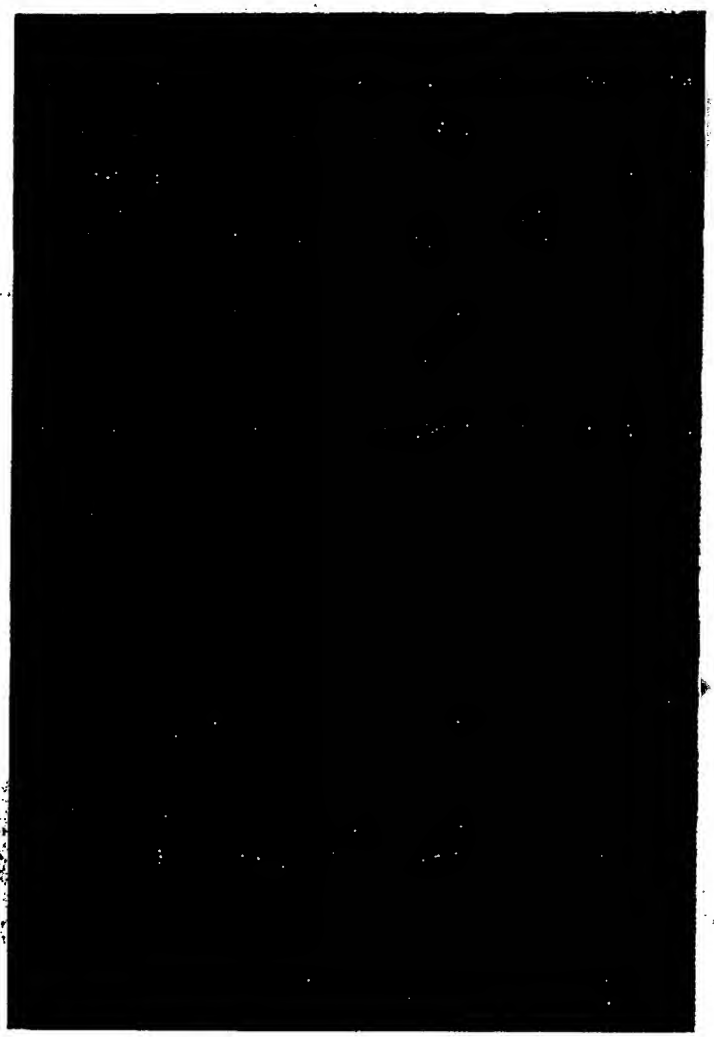


FIGURE 12

Negative reaction of Daudi/A9 clone OR cells (human-mouse hybrid clone) with EBV-negative serum as complement source and FITC-anti β_{1C}/β_{1A}.

FIGURE 13

Positive reaction of Raji EBV-positive serum, comp β_{1C}/β_{1A}.

the presence of EBV-antibodies in the sera tested here. Such a reaction was observed with all the human lymphoblastoid lines examined, regardless of origin or EBV-producer or non-producer status. Although every cell line has not been studied by DNA-hybridization or complement fixation tests on cell extracts, current experimental evidence obtained in several laboratories indicates that all established lymphoblastoid lines of human origin carry the EBV genome. Primate cell lines derived by transformation of peripheral lymphocytes by EBV were also reactive in the ACIF test. One cell line (1670) carrying HVS was non-reactive with five EBV-positive and five EBV-negative sera, while another line carrying HVS (MLC) reacted weakly with one of five positive sera. Other contr mouse origin and having with EBV did not react in factors indicated that the EBV-associated antigen lo of cells carrying EBV. Sta phase showed that the a with the chromosomes.

In contrast to EA and V antigen was present in ov cultures of all the huma lines tested, but the relati to other EBV-associate established. A likely can antigen would be the he fixing antigen which was and identical in producer lines (Walters and Pope, 1972). It was not estat

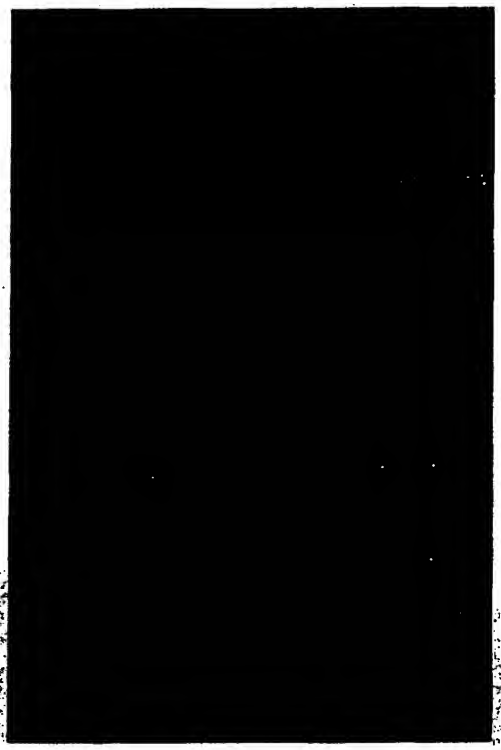


FIGURE 13

Positive reaction of Raji cell chromosomes with EBV-positive serum, complement and FITC-anti β_{1C}/β_{1A}.

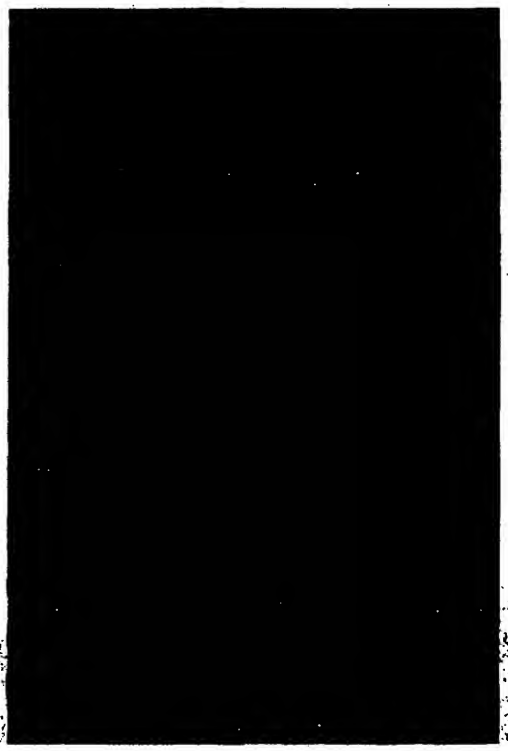


FIGURE 14

Non-EBV-related antinuclear reaction of Vero cells with serum (e) (Table VI), complement and FITC-anti β_{1C}/β_{1A}.

ouse hybrid
and FITC-

lymphoblastoid lines of
the EBV genome. Primate
ransformation of peripheral
' were also reactive in the
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positive sera. Other control cells of human and mouse origin and having no known association with EBV did not react in the ACIF test. These factors indicated that the ACIF test detected an EBV-associated antigen localized in the nucleus of cells carrying EBV. Staining of cells in metaphase showed that the antigen was associated with the chromosomes.

In contrast to EA and VCA, the nuclear ACIF antigen was present in over 90% of the cells in cultures of all the human lymphoblastoid cell lines tested, but the relationship of this antigen to other EBV-associated antigens was not established. A likely candidate for the nuclear antigen would be the heat-stable complement-fixing antigen which was shown to be present and identical in producer and non-producer cell lines (Walters and Pope, 1971; Reedman *et al.*, 1972). It was not established here that the nuclear antigen was identical from one cell line to another. Variations in intensity of the reaction from cell to cell or from smear to smear were partly attributable to technical factors but in some cases may have reflected a genuine variation in the amount of antigen produced. There were always some negative cells even in the best preparations. These cells may have been dead or in a non-reactive phase of the cell cycle.

Non-EBV-related antibodies to nuclear components have been demonstrated in sera from nasopharyngeal carcinoma patients (Yoshida, 1971) and infectious mononucleosis patients (Kaplan and Tan, 1968). Both entities are characterized by high levels of antibodies to EBV (Henle *et al.*, 1968; Niederman *et al.*, 1968; de Schryver *et al.*, 1969; Henle *et al.*, 1970) and EBV has been causally associated with heterophile-positive infectious mononucleosis (Henle

TABLE VI

DIFFERENTIATION OF EBV-RELATED ACIF REACTIONS AND NON-SPECIFIC ANTI-NUCLEAR REACTIONS OF SERA

Cell line [1]	Origin (cell type))	Reactions of sera in ACIF test [2]							
		EBV-negative [3] serum (complement source)	EBV-positive [3] sera		Sera [4] with non-specific antinuclear antibodies				
		2 (control)	32 (control)	49 (BL)	a (NPC)	b (NPC)	c (NPC)	d (CMV)	e (CMV)
Raji	human (lymphoblastoid)	− −	++	++	++	++	++	++	++
Fibroblasts	human (monolayer)	−	−	−	++	+	+	+	+
Normal glia	human (monolayer)	−	−	−	++	+	NT	NT	++
HBT	human breast cancer (monolayer)	−	−	−	++	++	+	+	+
Vero	green monkey kidney (monolayer)	−	−	−	++	+	+	+	++
MNC	marmoset (monolayer)	−	−	±	++	++	++	++	++
L cells	mouse (fibroblasts)	−	−	−	+	+		−	−

NT = not tested, BL = Burkitt's lymphoma, NPC = nasopharyngeal carcinoma, CMV = cytomegalovirus infection.
[1] See Tables II, III and IV.
[2] −, ±, + and ++ = negative, weak, strong and very strong nuclear staining.
[3] See Table I.
[4] Sera a, b, and c were previously tested by Dr. T. O. Yoshida, sera d and e by Dr. H. Thé for non-specific antinuclear antibodies. Code Nos: a = KCC 1249, b = KCC 1244, c = KCC 1245, d = 41 S9730, e = 39 S 9224.

et al., 1968; Evans *et al.*, 1968; Hampar *et al.*, 1971). Table VI clearly differentiates non-specific antinuclear reactions from the EBV-related reaction with Raji cells, although this does not exclude the possibility that some of the sera in Table I could have had non-specific antinuclear antibodies as well.

The ACIF test provides a sensitive method of tracing the EBV genome in cells where other EBV components are not readily detectable. In contrast to conventional complement fixation, this technique requires very few cells, and, under optimal conditions, should allow the differentiation of positive and negative cells in the one preparation. Thus, three clones of cells (OR, 10 and 9) derived by fusion of a lymphoblastoid cell line and a mouse fibroblast line had 60 to 80%, 20-25% and 1.5% reactive cells respectively, while a fourth clone was consistently non-reactive. These clones segregate in culture and preferentially lose human chromosomes. It is possible that particular human chromosomes or, alternatively, a critical minimum number of human chromosomes, must be present before the antigen can be detected. It is planned to combine ACIF, DNA-hybridization and chromosome studies as the clones segregate to determine which, if either, of these alternatives is correct. Since the antigen is specific for EBV, it follows that all or some of the essential chromosomes must carry the EBV genome.

In preliminary experiments, two Burkitt lymphoma biopsies also had nuclear antigens similar to those in the lymphoblastoid cell lines. These results with the biopsies are substantiated by the detection of EBV-specific complement-fixing antigens in cell extracts of several other biopsies (results to be published), and the demonstration by nucleic acid hybridization of the EBV genome in such biopsies (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1971 and unpublished results). Extension of these studies to other biopsies may help

EBV in human disease.

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to other biopsies may help clarify the role of EBV in human disease.

The nuclear ACIF reaction was obtained with EBV-positive sera and either fresh human or guinea-pig serum, and specific anticomplement reagents, and was inhibited by heat inactivation of the complement source. The reaction was therefore attributable to complement fixation. Presumably, the complement fixation step acted as an amplifier to increase the sensitivity of the immunofluorescence technique as in other systems (Hinuma and Hummeler, 1961; Hinuma *et al.*, 1962). There is no reason to assume that complement fixation was necessary for the antigen-antibody reaction. This reaction probably occurs in direct and indirect tests as well, but is not readily visualized.

The reaction of the complement alone with a small number of cells from some of the lymphoblastoid cell lines is in line with the findings of Floyd *et al.* (1971), who used guinea-pig complement in an ACIF test similar to that described here. The significance of this reaction is not understood. Since only a small minority of the cells reacted and since their appearance was entirely different from the specific nuclear reaction, it presents no problems in evaluating the EBV-associated ACIF test.

One non-lymphoblastoid cell line derived from a glioma contained a low frequency of cells (0.4-3.8%) giving a nuclear reaction with three of the EBV-positive sera, while a fourth serum reacted with 22% of the cells. The myeloma line RPMI No. 8226 contained a few cells giving nuclear staining with three EBV-positive sera but not with a negative serum. The cell lines derived from normal glia and sarcoma contained a very low level of cells (0.1-3%) reacting in each case with one of the positive sera only. Unlike the lymphoblastoid cell lines, the majority of cells in these cultures were non-reactive, and further work is required to determine if these reactions are EBV-related or not.

Since the nuclear ACIF antigen was present in most cells of the reactive cell lines, its presence is compatible with continued cell growth and multiplication. As such it is an antigen induced in non-permissive cells by EBV, in contrast to VCA and EA, which are produced during an infectious virus cycle leading to cell death. It is tempting to draw an analogy between this antigen and the T antigens of the papova virus systems. Further study of both types of antigens is potentially of importance in understanding the virus-cell interactions that are relevant for oncogenesis. We propose that the nuclear antigen in the lymphoblastoid cell lines be referred to as EBNA (EBV-associated nuclear antigen).

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LOCALISATION CELLULAIRE D'UN ANTIGÈNE ASSOCIÉ A L'EBV (VIRUS D'EPSTEIN-BARR) ET FIXANT LE COMPLÉMENT DANS LES LIGNÉES LYMPHOBLASTOÏDES PRODUCTRICES ET NON PRODUCTRICES

Les auteurs ont utilisé l'immunofluorescence anti-complément (ACIF) pour étudier les antigènes des lignées lymphoblastoïdes humaines qui fixent le complément. Ces lignées cellulaires portent le génome du virus d'Epstein-Barr (EBV), mais seules les cultures synthétisent des antigènes spécifiques de l'EBV (antigène des capsides virales (VCA) et antigène précoce EA)) décelables par immunofluorescence directe ou indirecte, et ce généralement dans moins de 5% des cellules. Le test ACIF a révélé la présence d'un antigène localisé dans le noyau des cellules lymphoblastoïdes. Contraire-

ment à l'EA et au VCA, cet antigène apparaissait dans plus de 90% des cellules des cultures productrices et non productrices. L'antigène est spécifique de l'EBV, comme le montre une comparaison des réactions de 52 sérums dans le test ACIF. Les sérums qui donnent une réaction nucléaire contiennent des anticorps contre le VCA, l'EA ou des antigènes décelables par des tests de fixation du complément sur des extraits cellulaires, mais les sérums dépourvus d'anticorps anti-EBV ne réagissent pas. Les auteurs ont constaté des réactions faibles, équivoques ou discordantes avec six sérums se caractérisant par un faible titre de VCA, d'EA ou d'antigènes décelés lors des tests de fixation du complément. Ils ont remarqué une réaction nucléaire dans les lignées cellulaires obtenues par transformation de lymphocytes d'humains et de primates par l'EBV. Les cellules témoins qui ne semblent pas associées à l'EBV ne réagissent pas. Il s'agissait de lymphocytes fœtaux transformés par la phytohémagglutinine, de lignées cellulaires dérivées de cancer du sein, de gliome, de névroglies normales, de pleurésie maligne et de myélome, ainsi que de deux lignées lymphoïdes de ouistiti porteuses du virus herpétique saimiri (HVS). Dans des expériences préliminaires, le test ACIF avait été utilisé pour dépister le génome de l'EBV au niveau cellulaire. Les cellules de deux biopsies de lymphome de Burkitt, les unes testées après la biopsie et les autres après passage sur des souris " nude ", contenaient un antigène spécifique de l'EBV. Trois clones de cellules dérivées d'hybrides de cellules somatiques de souris et d'une lignée lymphoblastoïde humaine contenaient aussi cet antigène, mais le nombre de cellules réactives variait d'un clone à l'autre. Un quatrième clone ne réagissait pas.

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Exhibit 3

CHAPTER 74

Epstein-Barr Virus and Its Replication

Elliott Kieff and Alan B. Rickinson

CLASSIFICATION

Epstein-Barr virus (EBV) was discovered in 1964 (192). Beginning in the 1940s, Denis Burkitt, a British missionary surgeon, observed and treated children with previously undescribed extranodal lymphomas. He noted that the lymphomas were frequent in regions of equatorial Africa with holoendemic malaria and rarely occurred elsewhere. Burkitt wrote and spoke widely about the unusual epidemiologic and clinical features of this lymphoma, raising the specter of an infectious etiology (81). After hearing Burkitt speak about this new disease at Bristol University, Tony Epstein arranged collaborations to obtain tumor biopsies and succeeded in culturing the lymphoma cells. Epstein, Achong, and Barr identified a herpesvirus in electron micrographs of the tumor cells growing in culture. They showed that the virus differed from the known human herpesviruses in being unable to replicate in other cultured cells and in being nonreactive with antibodies to other known human herpesviruses. EBV became the first candidate human tumor virus.

EBV is now the prototype of the gamma subfamily of potentially oncogenic herpesviruses. The gamma herpesvirus subfamily includes both the gamma 1, or *Lymphocryptovirus* (LCV), and gamma 2, or *Rhadinovirus* (RDV), genera. EBV is the only human LCV, and the recently discovered Kaposi's sarcoma-associated herpesvirus (KSHV) is the only human RDV (93). Herpesvirus saimiri (HVS), an RDV that was discovered in New World primates and found to cause lymphomas in experimental infection, was the previous prototype RDV (10). Many Old World primate species have their own endemic LCV, and recent evidence suggests that some New World primate species also have endemic LCVs (61,207,244,246,313,457,474,676). In contrast, RDVs have been identified not only in many primate species but also in many subprimate mammalian species (9,11,188, 858). At this stage of accumulation of LCV and RDV DNA sequences, RDV DNAs are more diverse than LCV DNAs. The endemicity of RDVs in a broader range of mammalian species and their greater genome divergence are evidence that RDVs evolved earlier than the LCVs. Given the many similarities of the LCV and RDV genomes and the restriction of LCVs to primates, LCVs are likely to have evolved from an early primate RDV. This would explain the presence of LCVs only in primates.

The LCV genomes are very similar to each other in structure and gene organization. In general, their DNAs are composed of colinearly homologous sequences. The EBV genome organization is shown in Figure 1 and compared to that of KSHV. The schematic diagram is based on the published analyses of KSHV DNA sequences. The LCVs share structural features such as similar 0.5-kbp

coimmunoprecipitated with EBNA-3C, EBNA-3C appeared to co-localize with ProT-α in nuclei (128). These data indicate that EBNA-3C could be involved in transcriptional effects through interaction with ProT-α.

EBNA-3A also represses the activity of the Cp promoter in B cells and in epithelial cells, and this activity largely depends on the domain through which EBNA-3A interacts with RBP-Jk (122). Using Gal-4 EBNA-3A fusion proteins and Gal-4-responsive promoters, the EBNA-3A repressive domain has been mapped to 143 amino acids that do not interact with RBP-Jk and can mediate down-regulation of transcription (71,122). EBNA-3A also has a potential weak activation domain that is evident when EBNA-3A lacking the amino acid 100-to-364 repressive domain are fused to the Gal-4 DNA binding domain and expressed in B lymphoblasts along with a Gal-4-responsive promoter (122). The activation effects appear to be B-cell specific and are not evident in epithelial cells. EBNA-3A used as bait in a yeast two-hybrid search for interacting proteins curiously retrieved the carboxyl part of the epsilon subunit of the chaperonin T-complex protein 1, and the p38 subunit of the aryl hydrocarbon receptor complex (401,402). EBNA-3A binds to p38 and causes p38 to partially localize to the nucleus (401).

EBNA-1

EBNA-1 was initially identified as an EBV nuclear neoantigen that is present in all EBV-infected cells, regardless of the state of EBV infection (682). This was the first evidence that EBV encoded a nuclear protein in latently infected cells, perhaps similar to T antigen in simian virus 40 (SV40)-transformed cells. Soon thereafter, EBNA-1 was shown to associate diffusely with mitotic chromosomes (625). EBNA-1 is unique among the EBNAs in this regard (274,652). The significance of EBNA-1 association with chromosomes emerged later from the discovery that EBV has a cis-acting element, termed oriP, that enables the persistence of episomes in EBV-infected cells or in any human cells in which EBNA-1 is expressed (906,910). EBV and other oriP-containing episomes associate randomly with human chromosomes in cells that express EBNA-1 (159,299, 753). The unique random association of EBNA-1 with chromosomes, the association of EBV or other oriP plasmid DNA with chromosomes in EBNA-1 expressing cells, and the need for EBNA-1 in oriP persistence, position EBNA-1 as the key mediator of EBV DNA binding to chromosomes and episome persistence. Indeed, EBNA-1 is essential for EBV episome maintenance (469).

OriP has at least two components: a family of 20 copies of a 30-bp repeat, FR, that can be an EBNA-1-dependent enhancer, and a dyad symmetry, DS, of four copies of the 30-bp repeat, two in tandem and two in a larger dyad symmetry, that are required for episome maintenance (683,684). A key component of the mechanism by which this system works is that EBNA-1 binds specifically to the 30-bp repeat (most strongly to the 20×30-bp repeat element) and then to the dyad symmetry element, and to another site within the EBNA transcript (681). An EBNA-1 expression vector and oriP can be combined with a cassette for positive selection to make a plasmid into which any gene can be cloned to enable its maintenance as an episome in human cells (784). The EBNA-1 and oriP system has been widely exploited to achieve heterologous gene expression. In one use, oriP enabled plasmids containing 150 to 200 kbp fragments of random human DNA to persist as multicopy episomes in EBNA-1-expressing cells. Two such plasmids could persist in the same cell (792). Although essential for oriP enablement of plasmid persistence, excess EBNA-1 expression does not increase oriP-replicated plasmid copy number. Cellular controls limit DNA replication to one initiation event per DNA molecule per S phase (2,909), and equal numbers of plasmids are distributed to cell progeny (787).

EBNA-1 is encoded by the 2-kb 3′-terminal exon of spliced mRNAs. In type 3 latency, the 3.5-kb EBNA-1 mRNA is initiated at the Cp or Wp promoter and is highly spliced, and the primary transcript is about 100 kb (see Fig. 3) (62–66,317,326,327,719,774,788). Transcription from the Wp promoter is initially regulated by cellular factors. Wp is up-regulated and Cp turned on by EBNA-2 and -LP. EBNA-1 then further up-regulates Wp and Cp. Eventually, high-level expression of EBNA-3s down-modulates the strong up-regulating effects of EBNA-2 and EBNA-LP and prevents runaway EBNA transcription (295,468,665,692,693,788,793,886). In latency type 1 or 2, the EBNA-1-specific 2.4-kb mRNA is primarily initiated at n62423, with secondary sites at 62392 and 62340 in the prototype B95 sequence (36,611–614,733). This latency type 1 and 2 Qp promoter lacks a recognizable TATAA element. Transcription appears to be positively regulated by an unknown factor, LBP-1 (611), by IRF1 and 2 (613,731), and by E2F (612,794), which bind to three distinct sites bracketing the n62423 initiation site. The two EBNA-1 binding sites just 3′ to the transcriptional start site are the principal elements that self-regulate EBNA-1 transcription in latency type 1 and 2 (720,732,794). Similar regulatory sites are conserved in the baboon LCV (711).

EBNA-1 from the prototypical B95 EBV strain consists of 641 amino acids. The protein has a high proline content, is charged, and migrates on denaturing polyacrylamide gels with an apparent size of 76 kd. From amino to carboxyl terminus, EBNA-1 has four components: (a) an amino-terminal 89 amino acids, which include amino acids 32 to 83 and are Arg rich, (b) amino acids 90 to 327, which are an irregular copolymer of Gly and Ala, (c) amino acids 328 to 386, which are Arg rich and include a

nuclear localization sequence at amino acids 379 to 386, and (d) carboxyl-terminal amino acids 387 to 641, which include amino acids 459 to 607 that bind DNA and dimerize. The two Arg-rich regions of EBNA-1 amino acids 1 to 379 can each bind to chromosomes and together reconstitute the chromosome association characteristic of EBNA-1 (360,532). Whereas EBNA-1 amino acids 379 to 641 appear to permit initial accumulation of oriP-containing plasmids in cells, the chromosome-associating domains are necessary for long-term plasmid maintenance, presumably because of their role in interacting with chromosomal proteins (349) and partitioning oriP episomes to progeny nuclei. Cellular chromosomal proteins, HMG-1 amino acids 1 to 90, or histone H1 can functionally substitute for EBNA-1 amino acids 1 to 378 in enabling long-term episome persistence (360).

Identification of the biochemical interactions of the EBNA-1 carboxyl-terminal domain with oriP is facilitated by the high expression level of the EBNA-1 carboxyl terminus in bacteria- or baculovirus-infected sf9 cells (230,574). EBNA-1 amino acids 450 to 641 or 607 have similar DNA binding and dimerization activity to full-length EBNA-1 (26,27,391,573,681). The core amino acids for DNA interaction are amino acids 459 to 500, particularly 462 to 477, whereas the core residues for dimerization are amino acids 501 to 532 and 554 to 598. A dimer of an EBNA-1 oligopeptide corresponding to amino acids 458 to 478 can bind DNA, albeit nonspecifically (99,100). Surprisingly, amino acids 450 to 641 fused to the pyruvate kinase ORF and the EBNA-1 nuclear localization signal (NLS) are sufficient for wild-type EBNA-1 transcriptional activation through FR upstream of a minimal TK promoter in Vero cells, and the carboxyl-terminal acidic domain is critical for activation (26).

Each 30-bp EBNA-1 dimer-binding site is a partial dyad similar to TAGGATAGCATATGCTACCCAGATCCAG (27,391,681). Relative to the center of each half of the dyad, nucleotides 3 to 8 are most critical for EBNA-1 binding (27). EBNA-1 has a high affinity for its cognate sequence, and interactions with the cognate sequence can be demonstrated even after protein denaturation and renaturation on cellulose nitrate. EBNA-1 not only dimerizes on 30-bp elements but also forms higher-order oligomers. Oligomerization of EBNA-1 on templates that have EBNA-1 binding sites separated by intervening sequence induces looping-out of the intervening DNA (230,259,543,781). On oriP, EBNA-1 first saturates FR and then binds to DS, looping out the intervening DNA (230). EBNA-1 amino acids 322 to 377 are important for efficient looping of EBNA-1 bound to FR and DS, or for linking multiple oriP DNA molecules to each other in a larger complex (228,518–520). Only DNA-bound EBNA-1 can participate in the large, linked EBNA-1 DNA complexes.

The binding of EBNA-1 to oriP results in two thymine residues, 64 bp apart in the region of dyad symmetry, becoming reactive to potassium permanganate, indicative of a helical distortion. EBNA-1 binding to DS lengthens interstrand H-bonds for three base-pairs centered over the permanganate-sensitive thymine base and three potential intrastrand H-bonds are formed between adjacent bases. Dimethyl sulfate protection studies indicate that EBNA-1 binds on the opposite face of the helix from the reactive thymines (229,307). Analyses on oriP in EBV-infected cells indicate that similar permanganate- and dimethyl sulfate–reactive sites exist *in vivo* (344).

Crystal structures of the EBNA-1 carboxyl-terminal 255-amino-acid dimer have been resolved at 2.5 Å, and of the dimer of cognate DNA at 2.4 and 2.2 Å (58–60). The EBNA-1 DNA binding domain has two structural motifs: a core domain that mediates protein dimerization and is similar to the DNA binding domain of the papillomavirus E2 protein, and a flanking domain that mediates sequence-specific contacts. Genetic and biochemical studies of the EBNA-1 core domain residues, which are structural homologs of the E2 residues that mediate sequence-specific DNA binding, implicate the corresponding EBNA-1 residues in DNA binding. The EBNA-1 core domain, when expressed in the absence of the flanking domain, has sequence-specific DNA binding activity, and the flanking domain residues contribute to the DNA binding. Thus, both the core and the flanking domains of EBNA-1 play direct roles in DNA recognition (132).

Only seven to eight copies of the 30-bp repeats are required for full activity of FR in transcription or episomal DNA replication (893). EBNA-1 binding to FR may effect up-regulation of the Wp and Cp promoters and of the far-upstream LMP1 and LMP2B promoters (241,665). The requirement for FR to enhance transcription and for episomal DNA replication can be partially replaced by a tandem duplication of DS (893). Tandem duplication of DS decreases its ability to serve as an origin (655). FR also arrests and functionally terminates the replication of episomes containing oriP in cells expressing EBNA-1 (167,240).

FR increases the retention and enhances transcription of oriP plasmids in cells, whereas DS is the site of initiation of plasmid DNA replication (300). DS supports EBNA-1-dependent replication in the absence of the FR (908). DNA synthesis begins in DS and proceeds bidirectionally from DS (240). Plasmids containing two copies of DS are not amplified relative to wild-type oriP (431). Two EBNA-1 binding sites in DS appear to be adequate for initial DNA replication (300,908). The ease of unwinding the 65-bp dyad symmetry of two EBNA-1 binding sites in DS, or relative nucleosome sparing in DS (i.e., relative to other sites in oriP) may contribute to the preferential use of DS as an origin (744,882). DS is not stringently required for episome maintenance, and other sequences can substitute for DS in initiation of DNA synthesis (109), including another site in oriP, designated

Rep* (432). DS is also a preferential but not exclusive site for EBV genome replication, and deletion of DS does not prevent EBV episome persistence (501,617).

At least one cellular cofactor that is not conserved in rodent cells is required for DS origin function and not for FR enhanced activity, as EBNA 1 enhances transcription in mouse or human cells, whereas oriP episomes do not replicate in rodent cells (893). Replication in rodent cells can be restored by replacing DS with a rodent DNA origin; such vectors are still dependent on the enhancer function of EBNA-1 and the family of repeats (447). Similarly, human cell DNA that contains a putative origin for DNA replication can functionally substitute for DS in oriP to allow persistence as an episome in human cells (448,542). A human cell cDNA has been identified that encodes a protein that interacts specifically with EBNA-1 cognate DNA, although the relevance of this protein to oriP function in DNA synthesis has not been established (924).

The ability of Rep*, human cell DNA origins, or rodent cell DNA origins to substitute for DS in the context of oriP in an EBNA-1-expressing cell, calls into question the fundamental role of EBNA-1 in DNA replication versus episome persistence (8). Indeed, oriP increases the conversion of transfected plasmid DNA to DpnI resistance about 10-fold in the absence of EBNA-1 at 48 hours after transfection and about 20-fold in the presence of EBNA-1. This DNA had gone through two cycles of DNA replication as evidenced by DpnI resistance and by bromodeoxyuridine incorporation. The major EBNA-1 effect occurred at 96 hours after transfection, at which point Dpn-resistant DNA disappeared from cells that lack EBNA-1. Further, DS was not specifically required for persistence; FR with a triplicate of Rep* DNA replicates and persists as well as wild-type oriP in cells that express EBNA-1.

EBNA-1 amino acids 90 to 327 are an irregular glycine-alanine repeat domain. The DNA encoding this domain is similar to repeat sequences in cell DNA, and similar polypeptides may be encoded from the cell genome (312). EBV isolates frequently differ in the length of this repeat. This difference has been useful in identifying the EBNA-1 ORF and typing EBV isolates (204,207-269,326). Deletion of the repeat does not affect EBNA-1 stability or function (907). EBNA-1 expressed in murine cells does not engender a specific cytotoxic T-cell response in mice (847). The failure to recognize EBNA-1 as foreign is due to the EBNA-1 Gly-Ala repeat, which inhibits EBNA-1 processing through proteosomes (477). Because proteosome-mediated degradation is necessary for antigen processing into major histocompatibility complex (MHC) class I molecules for presentation at the cell surface, epitopes in EBNA-1 are poor targets for attack by CD8 cytotoxic T cells. Fusion of the Gly-Ala domain to the EBNA-3B ORF down-regulates CD8 recognition of EBNA-3B-expressing cells. Other proteins are also protected from proteosome degradation by fusion to the glycine-alanine repeat (746). The length of the repeat and particularly the alanines are critical for this effect. Primate LCVs have shorter Gly-Ala repeats that do not have a similar effect on proteosome processing, leaving open the possibility that the Gly-Ala repeat domain may have yet another role in EBV infection.

EBNA-1 is very stable and is phosphorylated on serine residues in at least two separable domains in the carboxyl half of the molecule (308,652,658). The role of these phosphorylations in EBNA-1 function has not as yet been established through genetic analyses. Further, EBV DNA in human cells that express EBNA-1 associates with the nuclear matrix. The fragment of EBV DNA that associates with the matrix includes oriP, potentially linking oriP to a site for DNA replication or transcription. EBNA-1 can also interact with diverse nucleic acid substrates *in vitro*, including EBER-1 and HIV-TAR (768).

EBNA-1 is the only EBNA that continues to be transcribed when cells are activated to lytic infection (317,706,873,877). Cp and Wp promoter activities cease in lytic infection, and the downstream Fp promoter is activated (614). The lytic mRNA initiates in an Fp promoter just upstream of the Qp promoter and otherwise has the same sequence as the latent infection RNA.

LMP1

Despite being a weak promoter for the LMP1 gene relative to the Cp and Wp EBNA promoters, as revealed by nuclear run-on assays, LMP1 mRNA is almost 10 times more abundant than the EBNA mRNAs in latently infected B lymphocytes (212,720). In epithelial cells, LMP1 transcription is mediated by an upstream promoter that initiates transcription from multiple TATA-less sites in the nearest copy of TR, resulting in a 3.5-kb transcript with a long, largely untranslated 5′ exon (251,715). The LMP1 transcripts have two short introns (44,212). The EBNA proteins are remarkably stable, whereas a significant fraction of LMP1 has a short half-life. The primary amino acid sequence of LMP1 is that of an integral membrane protein and includes at least three domains: (a) a 20-amino-acid, arginine- and proline-rich, hydrophilic amino terminus lacking the characteristics of a signal peptide; (b) six markedly hydrophobic 20-amino-acid, alpha-helical, transmembrane segments, separated by five reverse turns, each eight to ten amino acids in length; and (c) a 200-amino-acid carboxyl terminus, rich in acidic residues (Fig. 8). LMP1, translated *in vitro*, posttranslationally inserts into cell membranes consistent with the expected membrane insertion properties of the three pairs of highly hydrophobic transmembrane segments joined by short reverse turns (490,492,724). Although LMP1 has little primary sequence homology to other proteins, aspects of its three-domain organization are similar to some other integral membrane proteins

CHAPTER 75

Epstein-Barr Virus

Alan B. Rickinson and Elliott Kieff

sion is suppressed and viral transcription is limited to the noncoding EBERs and possibly the BamHI A RNAs; this is equivalent to the Latency 0 form of infection postulated in Figure 1.

Analysis of lymphoid (mainly tonsillar) tissues from virus carriers suggests that the proportion of B cells that carry the virus here is again very low, similar to levels seen in the blood (11,24,330). *In situ* hybridization on tissue sections identifies occasional EBER-positive cells, again usually within extrafollicular areas, which have the appearance of small B lymphocytes and show no evidence of EBNA2 or LMP1 expression (17,330). A more complete histologic picture may be easier to discern, however, in the tonsils of virus carriers from areas where parasitic infection provides a chronic antigenic stimulus to the B-cell system. In such circumstances, recent work suggests that the extrafollicular load of EBER-positive small B cells is slightly elevated and that in some cases EBER-positive centroblasts, always EBNA2-negative but occasionally expressing LMP1, are detectable within germinal centres (17). Such findings would be consistent with a recent RT-PCR analysis of EBV gene transcription that has detected LMP1, LMP2A, and Qp-initiated EBNA1 mRNAs both in the germinal center B cell (CD19+, IgD--, CD77+) and in the memory B cell (CD19+, IgD--, CD77--) compartments of tonsillar lymphocytes (25,26). These appear to represent reactivations from the preexisting memory cell pool rather than *de novo* infections because such transcripts were consistently present in tonsils whether or not there was evidence of ongoing virus replication. Interestingly, Cp/Wp-initiated EBNA transcription, diagnostic of growth-transforming Latency III infection, was found only in tonsils in which virus replication was occurring to provide a source of progeny virus for *de novo* infections (25). Therefore, reactivations from the latently infected memory B-cell pool and *de novo* infections may be occurring simultaneously in the lymphoid tissues of virus carriers but are likely to involve different programs of virus gene expression and lead to different outcomes.

The spectrum of B-cell differentiation stages represented within lymphoid tissues is much broader than that represented in peripheral blood, and the resident forms of EBV infection are correspondingly more heterogeneous. EBV has evolved mechanisms to colonize, persist within, and reactivate from the human B-cell system that very probably exploit normal B-cell physiology, using differentiation-dependent cellular controls to activate or suppress different programs of viral gene expression at appropriate times in the life history of the infected cell. The full complexity of these viral strategies is only just being realized, and any current attempt to map the lineage, kinetic, and anatomic relationships between infected cells *in vivo* (see later overview section) must be regarded as preliminary.

ANTIBODY RESPONSES TO INFECTION

The seroepidemiology of EBV infection and of EBV-associated diseases relies heavily on a set of immunofluorescence assays developed in the laboratories of Werner Henle and George Klein within the first decade of EBV research (126). These measure antibody responses to the following serologically defined antigens: (a) the EBNA expressed in latently infected cells; (b) the early antigen (EA), divisible into diffuse (EA-D, methanol-resistant) and restricted (EA-R, methanol-sensitive) components and expressed within cells early in lytic cycle; (c) the virus capsid change (VCA) expressed within cells late in lytic cycle; and (d) the membrane antigen (MA) expressed on the surface of cells late in lytic cycle. As described in Chapter 74 (245), each of these "antigens" is a composite of several distinct viral proteins. Thus, EBNA is a complex of six distinct nuclear proteins, EBNA1, 2, 3A, 3B, 3C, and -LP, with antibodies to EBNA1 being the most frequent (but not the only) reactivity detected in the conventional anti-EBNA immunofluorescence test. EA is a plethora of immediate-early and early viral proteins, including the BZLF1 immediate early and the abundantly expressed BALF2, BHRF1, BMRF1, and BMLF1 early proteins. Much of what was termed VCA reactivity is diffuse cytoplasmic fluorescence and is directed against virus-encoded nucleocapsid components, including BcLF1, BFRF3, BLRF2, and the glycoprotein gp110, which are expressed in late lytically infected cells. On the other hand, most MA reactivity, detected by immunofluorescence staining of living cells, is directed against gp350, the most abundant viral protein present both on the surface of lytically-infected cells and on the viral envelope. Although less frequently assayed than anti-EA and anti-VCA, anti-MA levels in serum are of particular interest because they tend to correlate with virus neutralizing activity.

For clinical diagnostic work, attempts have been made to replace the above assays with enzyme-linked immunosorbent assay (ELISA)-based or other tests using as substrates either individual EBV proteins expressed in recombinant form or synthetic peptide mimics of immunodominant epitopes. Several such assays have been developed for the detection of primary EBV infection, based on IgM antibodies to the BALF2 and BMRF1 components of EA or to the low molecular weight VCA components BFRF3 and BLRF2. Screening on combinations of such antigens is still recommended for diagnostic accuracy (57,450).

Responses in Primary Infection

IM patients again provide the bulk of information available on the primary antibody response to EBV; what little is known about asymptomatic primary infec-

tions in young children suggests that their pattern of response is broadly similar (353). By the onset of clinical symptoms, most IM patients already have substantial titers of IgM antibodies to VCA and rising IgG titers both to VCA and to EA (most frequently, to the D component). IgA responses to these antigens may also appear transiently at this time, but this has not been systematically studied. The IgM anti-VCA response subsequently disappears either during convalescence or over the next few months, whereas IgG anti-VCA titers rise to a peak then may fall slightly over the ensuing months to a stable steady-state level. The IgG anti-EA response usually falls slightly faster and further than IgG anti-VCA and either becomes undetectable or stabilizes at a very low level (185). Virus neutralizing antibodies in acute IM tend to be of low titer and complement-dependent, consistent with antibodies to the major neutralization target antigen gp350 being predominantly of IgM class at this time, with IgG titers rising relatively late in the disease. A transient IgA response to gp350 is also detectable (430). Another characteristic and interesting feature of IM serology is the pattern of anti-EBNA reactivities. Many, but not all, patients in the acute phase of the disease show an IgG response to the EBNA2 protein (and probably also to the EBNA3A, 3B, 3C family of proteins, although these are more difficult to assay), whereas an IgG response to EBNA1 is not usually detectable until convalescence (190). Antibodies capable of recognizing EBNA1 in immunoblots have been detected in the IgM fraction of acute IM serum, but these are thought to be part of the autoantibody response (see later), with cross-reactivity against the glycine-alanine repeat domain of EBNA1 (372). Antibody responses to the latent membrane proteins LMP1 or LMP2 have not been seen in IM patients and indeed are only detectable in a small proportion of healthy carriers even using the most sensitive assays (139,306).

What role the humoral response plays in the overall control of the primary infection remains uncertain. Clearly, the virus-neutralizing components of the response (predominantly anti-gp350 antibodies) have the capacity to prevent generalized spread of the virus as a cell-free viremia; even here, however, the possibility has been raised that the IgA anti-gp350 response may actually facilitate virus spread to epithelial compartments (430). The delayed antibody response to EBNA1 remains difficult to understand. The original view, that this reflected the delayed destruction of latently infected cells *in vivo* and hence delayed availability of the antigen, is hard to reconcile with the observation that responses to EBNA2, another latent protein, are detectable during the acute disease (190), although they may not persist in the longer term. Possibly, the change in anti-EBNA reactivities between primary and persistent infection may reflect the fact that different types of latently infected cells predominate at these different times.

In addition to the previously described EBV-specific antibody responses, the early phase of acute IM is associated with generally elevated levels of total serum IgM, IgG, and IgA, again consistent with a virus-driven polyclonal activation of the B-cell system (185). Concomitant with this general increase is the transient appearance of a range of heterophile and autoantibodies, mostly of IgM class, which may also be products of virus-infected B cells (145). Of these various reactivities, heterophile antibodies with the capacity to agglutinate sheep and horse erythrocytes form the basis of the Paul-Bunnell-Davidsohn test that, when positive, is diagnostic of EBV-associated IM. Not all primary EBV infections become heterophile antibody-positive, however, and thus distinguishing EBV-associated IM from the IM-like illnesses caused by other agents, such as cytomegalovirus or toxoplasma, requires EBV-specific serology (185). The overall patterns of antibody responses seen during the acute and convalescent phases of IM are illustrated diagrammatically in Figure 3 relative to the virologic events (virus shedding in the throat, virus-infected B cells in the blood) occurring over the same time frame.

Responses in Persistent Infection

Healthy virus carriers are consistently IgG anti-VCA positive, anti-gp350 neutralizing antibody positive, and anti-EBNA1 positive; the serologic picture in convalescent IM patients also gradually changes to assume this same profile (126,185). Titers can differ markedly among individuals, and such differences tend to be stable over time. A significant number of long-term virus carriers possess additional reactivities, for instance, to EA (most frequently to the R component) or to one or more of the other EBNA proteins, and these again are stably maintained. The basis for these individual differences is not known. Healthy individuals with anti-VCA, anti-EA titers at the top of the normal range are not necessarily those showing the highest levels of virus replication, at least as measured by transforming virus titers in throat washings (507). Because antibodies to VCA, EA, and EBNA target intracellular antigen complexes, they seem unlikely to play a major effector role in recognizing and removing virus-infected cells *in vivo*. By contrast, anti-MA (anti-gp350) antibodies can bind to the surface of late lytically infected cells and also to virions, and they have the capacity to neutralize a broad spectrum of EBV strains. Such antibodies could therefore contribute to overall control of the established virus carrier state by preventing a recrudescence of viremia and, as discussed later, by helping to protect the immune host from superinfection with further strains of orally transmitted virus.

Exhibit 4

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Cytolytic CD4$^+$-T-Cell Clones Reactive to EBNA1 Inhibit Epstein-Barr Virus-Induced B-Cell Proliferation†

Sarah Nikiforow,[1] Kim Bottomly,[1] George Miller,[2]* and Christian Münz[3]

Departments of Immunobiology,[1] Pediatrics, Epidemiology & Public Health, and Molecular Biophysics & Biochemistry,[2] Yale University School of Medicine, New Haven, Connecticut 06520, and Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021-6399[3]

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In the absence of immune surveillance, Epstein-Barr virus (EBV)-infected B cells generate neoplasms in vivo and transformed cell lines in vitro. In an in vitro system which modeled the first steps of in vivo immune control over posttransplant lymphoproliferative disease and lymphomas, our investigators previously demonstrated that memory CD4$^+$ T cells reactive to EBV were necessary and sufficient to prevent proliferation of B cells newly infected by EBV (S. Nikiforow et al., J. Virol. 75:3740-3752, 2001). Here, we show that three CD4$^+$-T-cell clones reactive to the latent EBV antigen EBNA1 also prevent the proliferation of newly infected B cells from major histocompatibility complex (MHC) class II-matched donors, a crucial first step in the transformation process. EBNA1-reactive T-cell clones recognized B cells as early as 4 days after EBV infection through an HLA-DR-restricted interaction. They secreted Th1-type and Th2-type cytokines and lysed EBV-transformed established lymphoblastoid cell lines via a Fas/Fas ligand-dependent mechanism. Once specifically activated, they also caused bystander regression and bystander killing of non-MHC-matched EBV-infected B cells. Since EBNA1 is recognized by CD4$^+$ T cells from nearly all EBV-seropositive individuals and evades detection by CD8$^+$ T cells, EBNA1-reactive CD4$^+$ T cells may control de novo expansion of B cells following EBV infection in vivo. Thus, EBNA1-reactive CD4$^+$-T-cell clones may find use as adoptive immunotherapy against EBV-related lymphoproliferative disease and many other EBV-associated tumors.

B-cell lymphomas related to Epstein-Barr virus (EBV) occur in patients with immune deficiencies. Immune dysregulation, which predisposes patients to B-cell lymphomas and EBV-induced B-cell lymphoproliferative disease (LPD), may result from genetic abnormalities affecting lymphocytes (e.g., severe combined immunodeficiency, X-linked lymphoproliferative disease), viral infection of T cells (e.g., human immunodeficiency virus infection and AIDS), or immunosuppressive medical treatments directed against T cells, such as FK506, cyclosporine A, corticosteroids, depletion of T cells, or OKT3 T-cell-toxic antibodies (14, 21, 32, 62, 76). Whether lack of NK, of CD4$^+$, or of CD8$^+$ T-cell function allows for the outgrowth of EBV-infected B-cell lymphomas in these individuals remains unclear. Adoptive immunotherapy with polyclonal T-cell lines consisting of mixtures of CD4$^+$ and CD8$^+$ cells in various proportions can prevent EBV-induced lymphomas and LPDs that occur in the settings of bone marrow and solid organ transplantation (23, 26, 47, 75, 80, 81, 96).

Cytotoxic CD8$^+$ T cells that recognize latent EBV products, and perhaps lytic antigens, are likely to play a significant role in curtailing proliferation of EBV-transformed cells in vivo (28, 40, 49, 68). In comparison to the large numbers of EBV-reactive CD8$^+$-T-cell clones which have been generated, only a few CD4$^+$-T-cell clones which recognize EBV nuclear antigen 1 (EBNA1), EBNA2, BHRF1, and gp340 have been isolated (8, 25, 37, 50, 67, 78, 91, 92). However, recent findings emphasize the importance of CD4$^+$-T-cell effector function against EBV antigens (22, 60, 85, 94). Cytotoxic T-lymphocyte (CTL) lines consisting primarily of CD4$^+$ T cells have been effective in immunotherapy against LPDs (59, 84). Activated CD4$^+$ T cells derived from fresh peripheral blood mononuclear cells (PBMCs) can prevent outgrowth of newly infected B cells (57, 86). EBV-reactive CD4$^+$-T-cell lines have been shown to lyse lymphoblastoid cell lines (LCLs) via various mechanisms. However, the antigen specificity of all but one of the T-cell lines used in these experiments remains unknown (27, 93).

A significant proportion of memory CD4$^+$ T cells that recognize autologous LCLs are directed against the EBNA1 protein (43, 55). EBNA1 is expressed in every form of EBV-related malignancy, including posttransplant lymphomas. Tumors such as nasopharyngeal cell carcinoma, Hodgkin's lymphoma, and Burkitt's lymphoma (BL) that fail to express some or all of the dominant CD8$^+$-T-cell latent antigens still express EBNA1 (4, 15, 19). The EBNA1 protein contains a glycine-alanine repeat that prevents proper processing and presentation through the major histocompatibility complex class I (MHC I) pathway. Therefore, EBNA1 is poorly recognized by CD8$^+$ T cells (36, 44, 45, 56). Although CD8$^+$ CTLs have been raised against portions of EBNA1, these CTLs are unable to exert cytotoxicity against endogenous wild-type EBNA1 presented by LCLs (8, 9). However, several groups have recently cloned CD4$^+$ T cells, some of which recognize EBNA1 presented by LCLs and BL cells (37, 43, 61, 90).

Our laboratory recently demonstrated that CD4$^+$ T cells from healthy EBV-seropositive donors were necessary and suf-

* Corresponding author. Mailing address: Department of Pediatrics, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520. Phone: (203) 785-4758. Fax: (203) 785-6961. E-mail: george.miller@yale.edu.

† We dedicate the manuscript to Charles A. Janeway, Jr.

TABLE 1. HLA typing of LCLs

Cell line	MHC class I		MHC class II		
	A	B	DR	DRw	DQ
AB	2, 25	7, 8	7, 17	52, 53	2, 3
KB	1, 30	7, 37	13, 15	51, 52	1
AC	1, 29	35, 38	1, 13	52	1
BC	30, 32	13, 44	4, 7	53	2, 3
RD	2, 3	13, 51	7, 11	52, 53	2, 7
LG2	2	27	1		1, 5
CM	2, 68	7, 44	4, 15	51, 53	3, 6
CP	2, 3	60, 62	4, 17	52, 53	2, 3
PJ	3, 29	35, 44	1, 7	53	1, 2
JR	23	35	1, 17	52	1, 2
TY	10, 11	27, 60	4, 15	51	1, 7
RPMI6666	2, 3	7, 18	1, 5		6

ficient to prevent in vitro proliferation and early outgrowth of CD23-positive B cells newly infected by EBV (57). Several questions remained unanswered about the mixed $CD4^+$ effector cells present within the PBMC populations that limited B-cell proliferation. Were they directed against a specific EBV-encoded or -induced antigen? Was their recognition of infected cells mediated by MHC II presentation of viral peptides? How did they inhibit the early outgrowth of EBV-infected B cells? The availability of clonal populations of $CD4^+$ T cells directed against EBNA1 enabled us to establish the principle that a clonal $CD4^+$-T-cell population directed against a single EBV latent antigen can mediate early regression of infected B cells and allowed us to investigate their mechanism of action.

MATERIALS AND METHODS

Cell lines. Cell lines used included the EBV-transformed B-cell lines and EBV-positive Hodgkin's lymphoma cell line RPMI6666 listed in Table 1, the EBV-negative B-lymphoma line BJAB (39), and the EBV-positive marmoset cell line B95-8 (51). LCLs were generated by culturing PBMCs or CD3-depleted cells of healthy donors with supernatant of the B95-8 cell line in RPMI 1640 medium plus 10% heat-inactivated fetal bovine serum (FBS), 1 μg of cyclosporine A/ml or 10 nM FK506 (Fujisawa), and antibiotics. HLA haplotypes of LCLs, determined by serotyping at the Yale Organ Transplant and Immunology Laboratory, are displayed in Table 1.

Analysis of cell surface molecules. Viable cells were isolated on a Ficoll-Hypaque density gradient (ICN, Irvine, Calif.). Cells were washed and resuspended at 10^6 cells/ml in phosphate-buffered saline containing 5% FBS and 0.01% Na-azide. A mixture of saturating concentrations of two or three different fluorochrome-conjugated mouse monoclonal antibodies against human cell surface antigens was added for 45 min on ice. Cells were fixed in phosphate-buffered saline containing 1% paraformaldehyde. Antibodies included anti-CD3–fluorescein isothiocyanate (FITC), anti-CD4–phycoerythrin (PE), anti-CD8–PE, anti-CD8–Cychrome (Cyc), anti-CD14–PE, anti-CD19–PE, anti-CD19–Cyc, anti-CD23–FITC, and various anti-Vβ antibodies (gifts from D. Posnett, Cornell University, or purchased from Biosource International, Camarilla, Calif.). Isotype controls for background binding of the antibody molecules were a mixture of polyclonal murine immunoglobulin G1 (IgG1)-FITC, IgG1-PE, and IgG2-Quantum Red. Antibodies were purchased from Sigma (St. Louis, Mo.), Pharmingen (San Diego, Calif.), or DAKO (Carpinteria, Calif.). In the experiment illustrated below in Fig. 6, B-cell populations from two different donors were distinguished by the presence of HLA-B7 molecules on B cells from the HLA-DR7-negative donor and the absence of HLA-B7 molecules on B cells from the HLA-DR7-positive donor. The anti-HLA-B7–PE antibody was purchased from One Lambda (Canoga Park, Calif.). Staining was analyzed on a fluorescence-activated cell sorter (FACS; Becton Dickinson, Franklin Lakes, N.J.).

Intracellular staining. Cells were stained for perforin protein, Fas ligand protein, or cytokines using the Cytofix/CytoPerm kit (Pharmingen) after culture with MHC-matched LCLs and Golgiplug containing brefeldin A (Pharmingen). For detection of perforin, clones were stimulated with autologous or MHC I- or II-matched LCLs for 12 h. Brefeldin A (Sigma-Aldrich, St. Louis, Mo.) was added for the last 9 h of culture, and fixed cells were stained with PE-conjugated antiperforin murine antibody or control PE-conjugated polyclonal murine IgG2b antibody (Pharmingen). For detection of Fas ligand, clones were stimulated with autologous LCLs for 18 h; brefeldin A was added for the last 12 h of culture. Fixed cells were stained with a murine IgG isotype control or NOK-2 antibody directed against Fas ligand (Pharmingen) followed by staining with a FITC-conjugated antibody directed against the murine IgG constant region (Sigma). For detection of cytokines, cells were incubated with antibody to gamma interferon (IFN-γ; 1-D1K) and biotinylated antibody to interleukin-4 (IL-4; 12.1; MabTech, Nacka, Sweden) followed by incubation with anti-murine IgG–FITC antibody (Sigma-Aldrich) and avidin-Cyc (Pharmingen). After refixation in 1% paraformaldehyde, cells were analyzed by FACS.

RPAs. LCLs were seeded at 3×10^5/ml, and T-cell clones were seeded at 5×10^5/ml in culture medium with 20 ng of phorbol myristate acetate (PMA)/ml and 1 μM ionomycin (Calbiochem, San Diego, Calif.). Cells were harvested at 0, 6, 12, or 24 h after treatment with PMA and ionomycin. Total cellular RNA was isolated using the Qiashredder and RNeasy minikits (Qiagen, Valencia, Calif.). Cytokine (hCK-1/2) or apoptosis pathway (Apo-3d/4) template sets were labeled with [α-^{32}P]dUTP (NEN, Boston, Mass.) and hybridized with sample RNA using Riboquant RNase protection assay (RPA) reagents (Pharmingen). After an 18-h hybridization, protected RNA transcripts were separated on a 19:1 acrylamide-bis urea gel which was dried and exposed to Kodak X-OMat AR film.

DC preparation. PBMCs were isolated from venous blood by centrifugation at $500 \times g$ at room temperature over Ficoll-Hypaque lymphocyte separation medium (ICN). Positive selection for $CD14^+$ cells was performed using murine anti-human CD14 antibody-conjugated microbeads, MACS depletion ferromagnetic matrix columns, and a VarioMacs separating magnet (Miltenyi-Biotec, Auburn, Calif.). $CD14^+$ cells were grown in RPMI 1640 and 1% human plasma supplemented with penicillin, streptomycin, and amphotericin B (61). Recombinant human IL-4 and granulocyte-macrophage colony-stimulating factor were added to culture wells on days 0, 2, and 4 to final concentrations of 500 U/ml and 1,000 U/ml, respectively. At the time of maturation on day 6, nonadherent immature dendritic cells (DCs) were transferred to new plates. Human IL-1β, IL-6, tumor necrosis factor alpha (TNF-α) and prostaglandin E_2 were added to each well at final concentrations of 10 ng/ml, 1,000 U/ml, 10 ng/ml, and 1 μg/ml, respectively. After 48 h of culture in maturation medium, DCs were used or frozen. Cytokines were purchased from R&D (Minneapolis, Minn.); prostaglandin E_2 was purchased from Sigma.

Presentation of EBNA1 and other antigens by DCs. Antigens were introduced to DCs either as large proteins, small peptides, or through viral vectors. *Escherichia coli*-derived recombinant EBNA1 (rEBNA1; amino acids [aa] 458 to 641) or proliferating cell nuclear antigen (PCNA) were added at a concentration of 10 μg/ml to DC cultures immediately prior to maturation (7, 16, 97). EBNA1-derived peptides 15 to 20 aa in length were added at 1 to 10 μM to mature DCs in serum-free medium and incubated for 1 h at 37°C (61). In some experiments mature DCs were infected with a vaccinia virus containing an EBNA1 construct lacking the glycine-alanine repeat (vvEBNA1ΔGA) or with a control vaccinia virus (vvTK$^-$) at a multiplicity of infection of 2 (9, 36, 55, 56). Other mature DCs were infected with influenza virus X:31, A/Aichi/68 (H3N2) (Charles River Laboratories, North Franklin, Conn.) at an multiplicity of infection of 0.5. Virus and DCs were incubated for 1 h at 37°C in RPMI without serum and extensively washed in RPMI with pooled human serum prior to cocultivation with T cells.

Isolation and maintenance of $CD4^+$-T-cell clones reactive to EBNA1 and influenza virus. $CD4^+$ PBMCs were isolated from venous blood of three donors (BC, AC, and JB cell lines) using anti-CD4-conjugated microbeads and magnetic selection (Miltenyi-Biotec). BC $CD4^+$-T-cell lines were obtained after alternating stimulation with vvEBNA1ΔGA-infected DCs and autologous LCLs. DCs and LCLs were exposed to 3,000 and 20,000 rad, respectively, of gamma radiation in a ^{137}Cs irradiator (Gammacell 1000; Nordion Int. Inc., Ontario, Canada). Twice, the line was enriched for EBNA1-reactive $CD4^+$ T cells to a frequency of 1.5% using magnetic beads which isolated IFN-γ-secreting cells (Miltenyi Biotec) (61). AC $CD4^+$-T-cell lines were obtained after two rounds of exposure to DCs pulsed with EBNA1 aa514-527 peptide. JB $CD4^+$-T-cell lines were obtained by multiple rounds of stimulation with influenza virus-infected DCs and one enrichment via an IFN-γ secretion assay. Clonal T cells were obtained by seeding enriched populations at 10, 1, and 0.3 cells per well with 10^5 gamma-irradiated PBMCs (3,000 rad), 2×10^3 gamma-irradiated LCLs (20,000 rad), 150 U of IL-2 (Chiron, Emeryville, Calif.)/ml, and 1 μg of phytohemagglutinin (PHA)/ml in RPMI plus 8% pooled human sera (Labquip Ltd., Niagara Falls, N.Y.). Wells containing proliferating cells which originally were seeded with 1 or 0.3 T cells were tested in split-well enzyme-linked immunospot (ELISpot) assays for antigen

specificity. Clones were expanded from frozen stock by cocultivation with irradiated PBMCs and LCLs, IL-2, and PHA. Clones were maintained by replacing half of the culture medium with fresh medium containing 300 U of IL-2/ml weekly. Approximately every 3 to 4 weeks, BC and AC clones were restimulated with gamma-irradiated autologous LCLs; the JB.flu10 clone was restimulated with autologous influenza virus-infected gamma-irradiated DCs.

Assessing the specificity of EBNA1- and influenza virus-reactive CD4$^+$-T-cell clones. The antigen specificity of BC clones was verified in ELISpot assays by response to DCs infected with vvEBNA1ΔGA versus that of DCs infected with vvTK$^-$, the vector control, and by response to DCs loaded with rEBNA1 versus DCs loaded with PCNA. Antigen specificity of the AC clones was verified by their response to DCs loaded with rEBNA1 versus that of DCs loaded with PCNA or the response to DCs loaded with EBNA1 aa 514 to 527 versus that of DCs loaded with EBNA1 aa 481 to 500 or aa 551 to 570. Antigen specificity of the JB clone was verified by response to DCs infected with influenza virus versus that of DCs infected with vvTK$^-$.

Maintenance and stimulation of an EBNA3A-reactive CD8$^+$-T-cell clone. CD8$^+$ PBMCs were isolated from venous blood of an HLA-B8$^+$ donor (MS cell line) using anti-CD8-conjugated MACS microbeads and magnetic selection (Miltenyi-Biotec). A CD8$^+$-T-cell line was obtained after 2 weeks of stimulation with autologous LCLs. The line was enriched for EBNA3A-reactive CD8$^+$ T cells over anti-PE MACS magnetic beads (Miltenyi-Biotec), which isolated cells bound to EBNA3A aa 325 to 333, HLA-B8–PE–conjugated tetramers (1, 12). Clonal CD8$^+$ T cells were obtained by limiting dilution. Their antigen specificity was tested in split-well ELISpot assays. The MS.B11 clone was activated by exposure to HLA-B8-positive LCLs pulsed with 1 μM FLRGRAYGL peptide (10, 18, 34, 52, 64).

ELISpot assays for cytokine-secreting cells. MAHA S45 plates (Millipore, Bedford, Mass.) were coated with antibody to IFN-γ (1-D1K), antibody to IL-4 (82.4), or antibody to IL-5 (TRFK5) (MabTech) at 5 μg/ml in sodium bicarbonate buffer, pH 9.5. From 5×10^3 to 1×10^5 T cells were added per well. Freshly EBV-infected or mock-inoculated B cells, EBV-transformed LCLs, or DCs loaded with EBNA1 or control proteins were added to the T cells. Cells were cocultivated for 18 to 24 h in RPMI plus 5% pooled human sera, washed, and incubated with a biotinylated secondary antibody; these included antibody to IFN-γ (7-B6-1), antibody to IL-4 (12.1), and antibody to IL-5 (5A10) (MabTech). Plates were washed and incubated with a preassembled avidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.). Spots left by cytokine-secreting cells were developed by addition of stable diaminobenzidine (Invitrogen, Carlsbad, Calif.). Spot-forming cells (SFCs) per well were counted using a stereomicroscope.

Antibody blocking of MHC interactions. Anti-HLA-DR antibody L243 (42, 61) and anti-HLA-A, -B, and -C antibody w6/32 (6, 61) were isolated from hybridoma supernatant passed over a protein A column; the IgG fraction was eluted with 50 mM glycine, pH 3. The anti-HLA-DR antibody LB3.1 was a gift from Jordan Pober (20, 74). LCLs, or B cells 4 to 5 days after infection with EBV, were exposed to 25 to 100 μg of MHC-specific antibody/ml for 1 h at 37°C prior to cocultivation with T cells.

Assay for EBV-induced B-cell lymphoproliferation and T-cell control. EBV and mock inocula were prepared as described previously from culture supernatants of the EBV-positive B95-8 cell line and the EBV-negative BJAB cell line, respectively (39, 51, 57). Subpopulations of PBMCs were selected as described previously (57). CD3-depleted populations were negatively selected using magnetic beads conjugated to murine anti-human CD3 antibodies (Dynal, Lake Success, N.Y.). Purified B cells were isolated by two steps of negative selection: all CD3$^+$ cells were removed, and then the Dynal B-cell-negative isolation kit was used to deplete all remaining non-B cells. CD4$^+$ T cells were positively selected on anti-CD4 antibody-conjugated microbeads and MACS selection columns (Miltenyi-Biotec). These selected populations and the desired concentrations of T-cell clones were then exposed to mock or EBV inoculum and cultured for 4 to 5 days for use in ELISpot assays or cocultivated for 16 to 18 days and analyzed for CD23 expression in regression assays. The number and percentage of CD19$^+$ CD23$^+$ cells were used as markers of immune control over EBV-exposed B cells (3, 11, 29, 57, 87).

Cytotoxicity assays. A total of 10^7 LCLs/ml were labeled with 2.5 to 5 μg of calcein (Molecular Probes, Eugene, Oreg.)/ml (71, 89). Labeled targets were incubated from 3 to 19 h with T-cell clones in RPMI plus 10% FBS without phenol red. Fluorescence retained in the cell pellet was detected on a CytoFluor II (PerSeptive Biosystems, Framingham, Mass.) with excitation at 480/25 nm and emission at 530/35 nm. Percent specific killing was calculated as follows: [(fluorescence maximal retention − fluorescence experimental well)/(fluorescence maximal retention − fluorescence minimal retention)] × 100. Maximal retention was determined by incubating the labeled targets alone or with MHC II-mismatched influenza virus-reactive CD4$^+$ T cells. Minimal retention was determined by incubating targets in a lysis buffer of 50 mM sodium borate, 0.1% Triton X-100, pH 9. Where indicated, T-cell clones were incubated with 10 μg of anti-Fas ligand antibody NOK-2 (Pharmingen)/ml, 10 μM brefeldin A (Sigma-Aldrich), or 100 nM concanamycin A (Fluka, Milwaukee, Wis.); LCLs were incubated with 250 ng of anti-Fas antibody ZB4 (Upstate Biotechnology, Lake Placid, N.Y.)/ml or 100 μM pan-caspase inhibitor zVAD-fmk (Molecular Probes) for 2 h at 37°C prior to mixing the targets and T cells in culture. Transwell cytotoxicity experiments were conducted in 24-well plates containing inserts with a bottom membrane containing 3-μm pores to allow exchange of media and soluble factors (Transwell polycarbonate membrane; Costar, Cambridge, Mass.). Calcein retention from triplicate samples was assessed for each set of culture wells.

RESULTS

CD4$^+$-T-cell clones raised against EBNA1 demonstrate a Th0 phenotype. Our earlier experiments showed that mixed populations of CD4$^+$ T cells controlled the activation and proliferation of B cells newly infected by EBV (57). The goal in the present experiments was to investigate the potential for CD4$^+$-T-cell clones directed against a single EBV latency antigen, EBNA1, to establish immune control over the proliferation of B cells freshly infected with EBV. The use of cloned T cells with a single antigen specificity ensured that cells which recognized EBV antigens were mediating immune regression.

We studied the activity of two groups of CD4$^+$-T-cell clones: one group of three clones was derived from an individual (BC) by alternating exposure to DCs expressing vvEBNA1ΔGA (aa 1 to 92, aa 323 to 641) (9) and autologous LCLs; a second group of two clones was derived from a different individual (AC cell lines) by exposure to DCs pulsed with a 14-aa EBNA1 peptide (aa 514 to 527) known to bind to HLA-DR1 (37, 38). All five clones were CD3 positive, CD4 positive, and CD8 negative (Fig. 1A and data not shown). FACS analysis of the T-cell antigen receptors showed that each BC clone expressed a single Vβ chain and that Vβ usage was unique to each clone (BC.E112, Vβ2; BC.E122, Vβ8; BC.E160, Vβ13.1) (61). Over months of repeated stimulation with MHC-matched LCLs or PHA, all the clones remained preferentially reactive to rEBNA1 (amino acids 458 to 641) provided to MHC II-matched DCs at the time of maturation (Fig. 1B and data not shown).

In order to characterize any effector functions of the EBNA1-reactive CD4$^+$-T-cell clones which might enable them to enact immune control over EBV infection, we analyzed their cytokine profiles. Although these clones were isolated for their ability to express IFN-γ in response to EBNA1, upon activation they expressed other Th1 cytokines and varying levels of Th2-type cytokine RNA transcripts and proteins (Fig. 2 and data not shown). Transcription of IL-2, IFN-γ, TNF-α, TNF-β, and transforming growth factor β1, classic Th1 cytokines, was up-regulated in clone BC.E112 after stimulation with PMA and ionomycin. However, exposure to PMA and ionomycin also induced transcripts for the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 (data not shown). Upon incubation with autologous and MHC II-matched targets, all BC clones secreted IL-4 and IL-5 at levels detectable by ELISpot in addition to large amounts of IFN-γ. However, the pattern of cytokine production by the BC.E112 and BC.E122 clones differed from that of clone BC.E160. In one experiment, clone BC.E112 yielded over 600 IFN-γ-secreting cells when stimu-

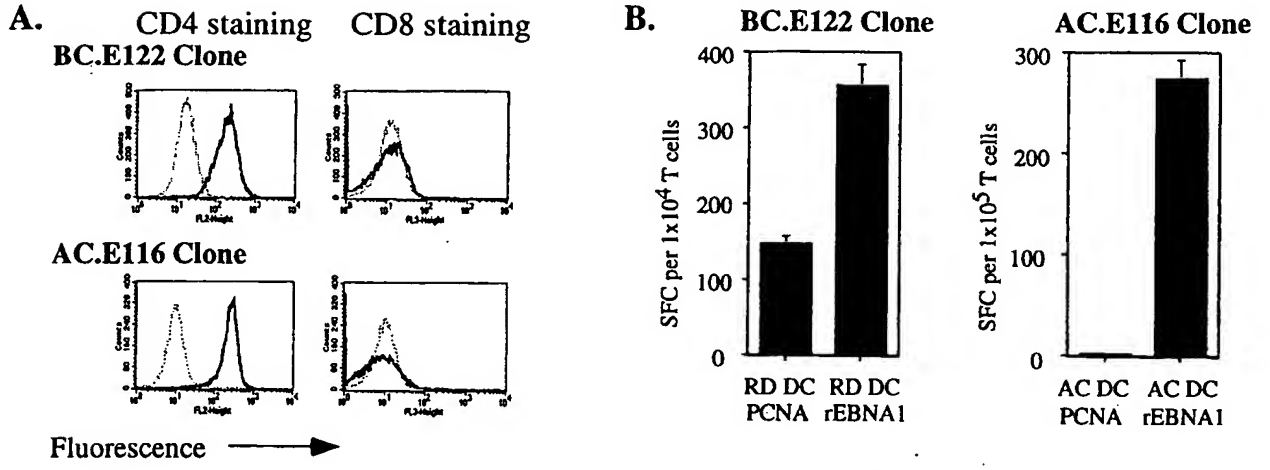


FIG. 1. CD4+-T-cell clones recognize EBNA1 presented on MHC II-matched DCs. (A) FACS analysis of T-cell clones incubated with fluorochrome-conjugated antibodies against CD4 (left panels; thick lines), CD8 (right panels; thick lines), or isotype control antibodies (thin, dotted lines). (B) IFN-γ secretion by a representative BC clone, BC.E122, and a representative AC clone, AC.E116, in response to DCs loaded with *E. coli*-derived rEBNA1 (aa 458 to 641) or PCNA proteins. HLA-DR7-matched (BC.E122 panel) or autologous (AC.E116 panel) DCs were loaded with protein at the time of maturation and seeded in ELISpot wells. Results represent the average of triplicate samples; standard errors of the means are shown.

lated with autologous LCLs, whereas only 23 BC.E112 cells secreted IL-4. In contrast, more than 260 cells from clone BC.E160 secreted IL-4 or IL-5 when stimulated with autologous or MHC II-matched LCLs in ELISpot assays (Fig. 2A and data not shown).

By staining for intracellular cytokines, we showed that activation with autologous LCLs led to production of IL-4 alone, IFN-γ alone, and IL-4 and IFN-γ together by BC.E160 cells (Fig. 2B). Global activation with PMA and ionomycin also induced many BC.E160 cells to secrete both IL-4 and IFN-γ. As individual cells concurrently secreted Th1- and Th2-type cytokines, the BC.E160 clone had a Th0 phenotype (24, 48, 63, 72). IFN-γ secretion by the cloned cells was used to assess the MHC restriction of activity in the following experiments.

EBNA1-reactive CD4+-T-cell clones recognize LCLs in an MHC class II-restricted manner. A panel of LCLs was used to

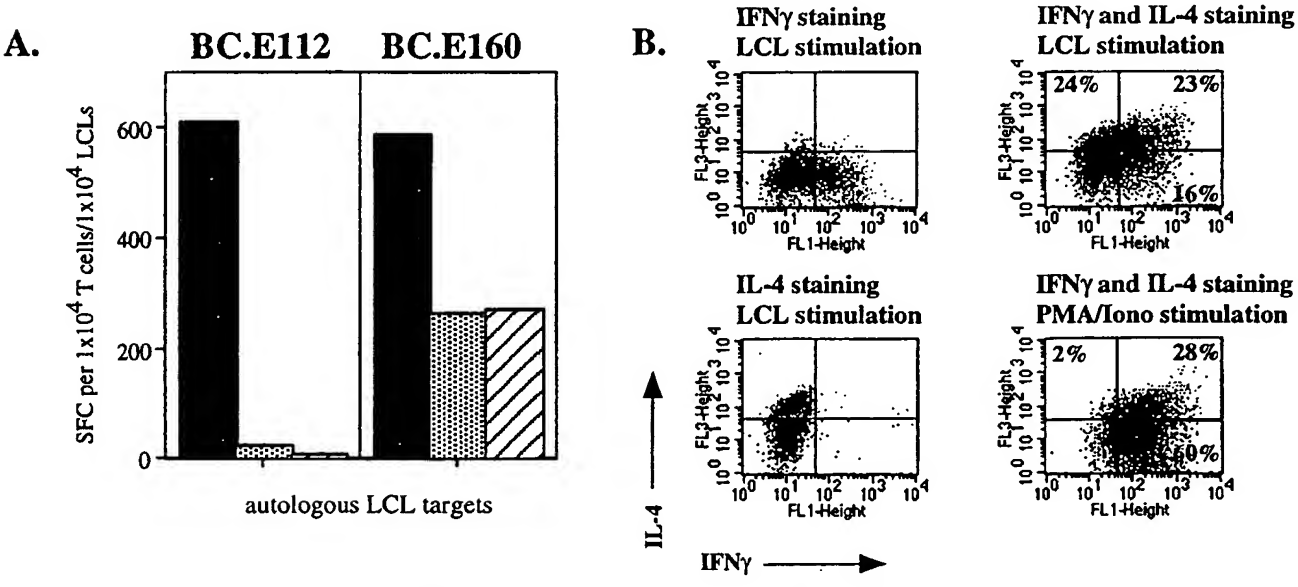


FIG. 2. CD4+-T-cell clones raised against EBNA1 express both Th1- and Th2-type cytokines. (A) Secretion of IFN-γ (solid bars), IL-4 (stippled bars), and IL-5 (hatched bars) by 10^4 BC.E112 (left panel) or BC.E160 (right panel) cells in response to 10^4 autologous LCLs in ELISpot assays. SFC counts represent the average of duplicate samples. (B) Intracellular staining for production of IFN-γ (FL1 fluorescence) or IL-4 (FL3 fluorescence) by the BC.E160 cells. The BC.E160 clone was stimulated for 18 h with PMA and ionomycin or autologous LCLs. Plots on the left represent cells stained with antibody to IFN-γ alone (upper left plot, lower right quadrant) or antibody to IL-4 alone (lower left plot, upper left quadrant). Plots on the right represent cells stained with antibodies to both IFN-γ and IL-4 (upper right quadrants).

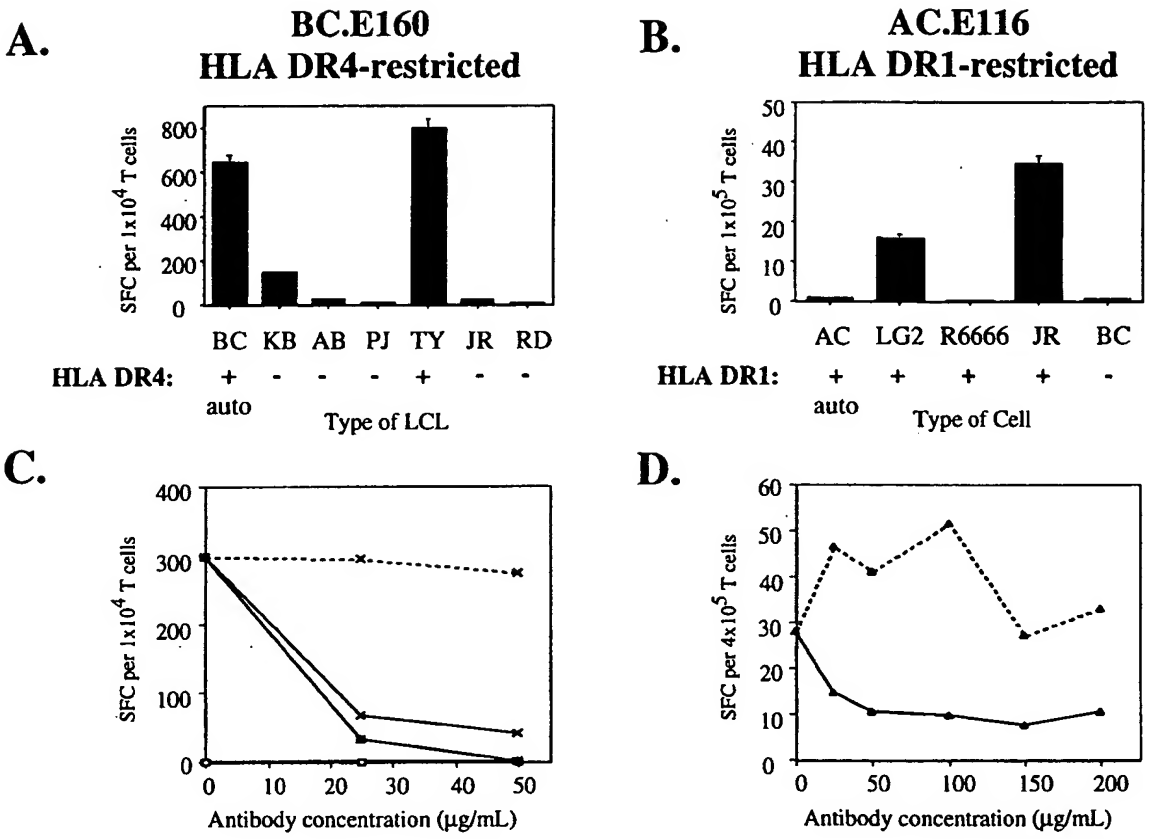


FIG. 3. EBNA1-reactive CD4$^+$-T-cell clones recognize MHC II-matched LCLs. (A and B) Recognition of LCLs bearing different MHC II molecules by EBNA1-reactive CD4$^+$-T-cell clones in IFN-γ ELISpot assays; 10^4 BC.E160 cells were added to 10^4 LCLs from donors matched or mismatched for the HLA-DR4 molecule (A), or 10^5 AC.E116 cells were added to 10^5 LCLs or an HLA-DR1-positive Hodgkin's lymphoma (RPMI6666) (B). SFC counts represent the average of triplicate samples; standard errors of the means are displayed. (C and D) Effect of antibodies against MHC I and MHC II on IFN-γ secretion by clones incubated with MHC II-matched LCLs in ELISpot assays. Antibodies to MHC I molecules (dotted lines), to MHC II molecules (solid line, open symbols), or a mixture of antibodies to MHC I and II (solid lines, filled symbols) were added at the indicated concentrations to LCLs and clones. LCL targets were HLA-DR4$^+$/DR7$^-$ (x's) added at 5×10^3 per well to BC.E160 cells (C) or HLA-DR1$^+$ (triangles) added at 5×10^4 per well to AC.E116 cells (D). SFCs represent the average of duplicate samples; data are representative of at least two experiments performed with each set of clones.

identify B cells which could serve as MHC-matched targets in regression assays. Each of the three clones in the BC series recognized the autologous LCL and LCLs expressing matched MHC II molecules (Fig. 3A and data not shown). Clones BC.E112 and BC.E122 secreted IFN-γ in response to LCLs that expressed HLA-DR7, whereas clone BC.E160 secreted IFN-γ in response to LCLs that expressed HLA-DR4. The two clones in the AC series recognized two LCLs that expressed HLA-DR1 but failed to recognize two other HLA-DR1-positive cell lines, including the autologous LCL (Fig. 3B and data not shown). Moreover, the number of IFN-γ-secreting cells among the AC.E116 cells was at least 10-fold lower than the number reacting among the BC clones to MHC II-matched LCLs, despite the addition of 10-fold more LCLs and 10-fold more T cells per ELISpot well.

To explore further the role of MHC molecules in antigen recognition by the CD4$^+$ clones, we carried out blocking experiments using antibodies directed against MHC I and MHC II molecules. The ability of all three BC clones to recognize MHC II-matched LCL targets was markedly inhibited by addition of the L243 or LB3.1 antibodies directed against HLA-DR molecules (Fig. 3C and data not shown). By contrast, addition of w6/32 antibodies against MHC I had little inhibitory effect. The combination of antibodies to MHC I and II together was not significantly more inhibitory than antibodies to MHC II alone. Antibodies to MHC II, but not to MHC I, also blocked the reactivity of clone AC.E116 to an HLA-DR1-matched LCL (Fig. 3D). In summary, IFN-γ secretion in response to a panel of LCLs and the blocking effects of antibodies confirmed that the CD4$^+$-T-cell clones recognized antigen in conjunction with specific MHC II molecules.

CD4$^+$-T-cell clones reactive to EBNA1 recognize B cells newly infected by EBV in an MHC II-restricted manner. In the previously described experiments we explored the ability of CD4$^+$-T-cell clones to recognize EBV-transformed LCLs. LCLs differ dramatically in surface markers, adhesion molecules, and viral protein expression from the heterogenous population of B cells found in the early stages of infection by EBV (3, 5, 66, 88). Therefore, we investigated whether the same EBNA1-reactive clones that recognized LCLs also recognized B cells freshly infected with EBV.

The number of IFN-γ-secreting cells among the BC.E112

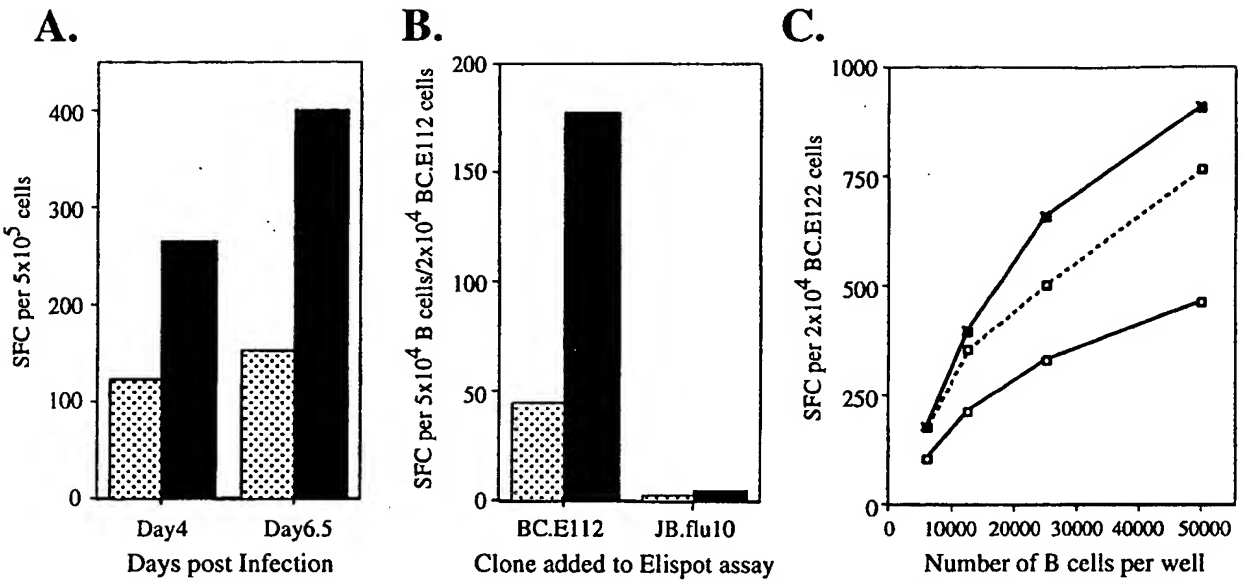


FIG. 4. EBNA1-reactive CD4+-T-cell clones secrete IFN-γ in response to B cells newly infected with EBV. (A) IFN-γ secretion in an ELISpot assay by a 2:1 mixture of clone BC.E112 cells and freshly EBV-infected (solid bars) or mock-inoculated (stippled bars) B cells after coculture for 4 and 6.5 days. (B) IFN-γ secretion by clone BC.E112 and clone JB.flu10 cells exposed to B cells for the 18-h duration of an ELISpot assay. The B cells had been cultured with EBV (solid bars) or a mock inoculum (stippled bars) for 4 days prior to exposure to the clones. Results represent the average of duplicate samples and are representative of at least two experiments performed on B cells from different donors. (C) IFN-γ secretion by clone BC.E122 in response to purified B cells cultured with EBV for 4 days. B cells were incubated with antibodies against MHC I or II at 100 μg/ml before addition of the T-cell clones. No antibody addition (solid line, filled symbols), addition of antibody to MHC I (dashed line, open symbols), and addition of antibody to MHC II (solid line, open symbols) are represented.

(Fig. 4A and B) and BC.E122 (Fig. 4C and data not shown) clones were two to three times greater in the presence of EBV-infected B cells than in the presence of B cells exposed to a mock inoculum. Cells from clones BC.E112 and BC.E122 secreted IFN-γ in response to B cells that had been infected with EBV for only 4 days. This reactivity to early EBV infection was observed when the BC.E112 clone was cocultured with the B cells from the time of infection (Fig. 4A) as well as when the B cells were infected separately for 4 days and then mixed with the BC.E112 clone for the 18-h duration of the ELISpot assay (Fig. 4B). Cells of a CD4+-T-cell clone directed against influenza virus, JB.flu10, did not respond to EBV-infected or mock-inoculated B cells, although the clone was capable of secreting IFN-γ in response to influenza virus-infected DCs (Fig. 4B and data not shown). The IFN-γ response of the BC clones to freshly EBV-infected B cells was mediated primarily through MHC II molecules. In ELISpot assays, antibody to MHC II decreased the number of IFN-γ-secreting BC.E112 and BC.E122 cells much more effectively than did antibody to MHC I (Fig. 4C and data not shown).

CD4+-T-cell clones raised against EBNA1 prevent proliferation and early outgrowth of EBV-infected CD23+ targets derived from the autologous donor. The capacity of memory T cells from an EBV-seropositive donor to prevent the outgrowth of EBV-infected cells is termed regression (41, 53, 54, 69, 70). In previous work our group demonstrated that an early event in regression is the capacity of a mixed population of memory CD4+ T cells to inhibit proliferation of EBV-infected CD23+ B cells (57). Since CD23 synthesis is regulated by the EBV latent protein EBNA2, a transcription factor essential to B-cell immortalization, CD23 expression correlates with transformation of B cells by EBV (3, 11, 29, 87). Depletion of CD3+ T cells or CD4+ T cells, but not of CD8+ T cells, resulted in the accumulation of CD23+ B cells 10 to 21 days after EBV infection (57). A mixed population of CD4+ T cells isolated from a seropositive donor prevented the accumulation of CD23+ B cells when added to CD3-depleted EBV-infected targets.

The antigen specificity of these polyclonal CD4+ T cells was likely to be diverse and could include a multitude of unknown viral and cellular targets. Therefore, we asked whether CD4+-T-cell clones reactive to EBNA1, and capable of secreting IFN-γ in response to newly EBV-infected B cells, would also enact regression. Addition of the BC.E122 (HLA-DR7-restricted) or BC.E160 (HLA-DR4-restricted) clone to CD3-depleted targets from the autologous donor at the time of EBV infection in vitro markedly diminished both the percentage and number of CD23+ B cells remaining after 18 days in culture (Fig. 5A and B). The numbers of CD23+ B cells remaining after addition of T-cell clones at a clone-to-target cell ratio of 1:1 were similar to those remaining in the mixed PBMC cultures. Based on titration, the BC.E122 clone was slightly more effective than the BC.E160 clone at inducing regression. The capacity of the clones to enact regression decreased as fewer T cells were added, but significant effects on the number and percentage of CD23+ B cells remaining after 18 days were evident even at a T cell-to-target cell ratio of 1:100.

Neither AC clone was capable of enacting regression in a culture of autologous EBV-infected CD3-depleted cells; however, mixed CD4+ T cells from the AC donor mediated re-

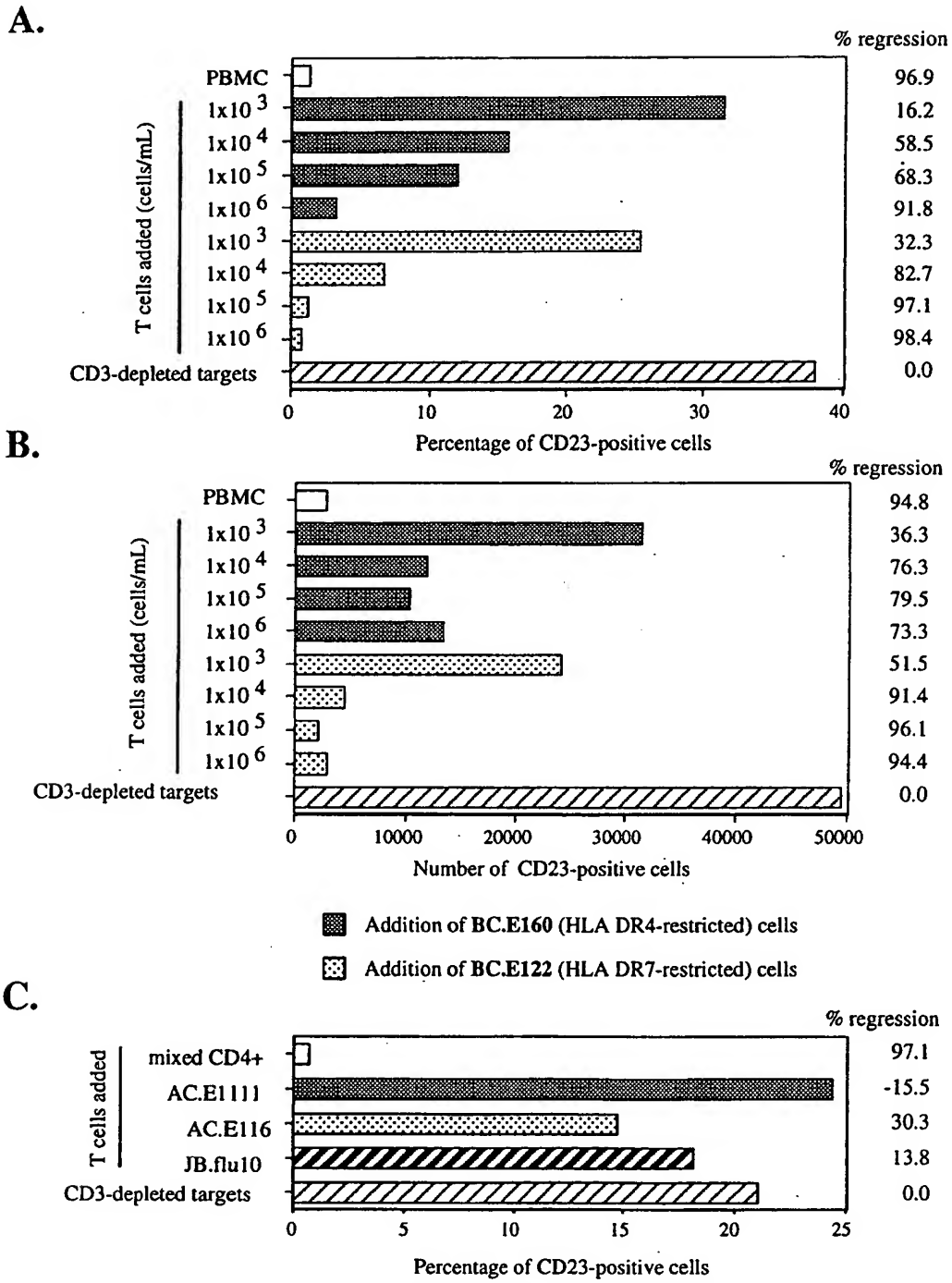


FIG. 5. EBNA1-reactive $CD4^+$-T-cell clones from donor BC reduce outgrowth of $CD23^+$ B cells in EBV-infected cultures derived from the autologous donor. Shown are the percentage (A and C) and number (B) of $CD23^+$ B cells remaining after 16 to 18 days in culture. (A and B) Serial 10-fold dilutions of two clones, the HLA-DR4-restricted BC.E160 (gray bars) and the HLA-DR7-restricted BC.E122 clone (stippled bars), were added to 10^6 EBV-infected CD3-depleted PBMCs/ml from autologous donor BC cells. Hatched bars represent the outgrowth of CD3-depleted target cells without the addition of T-cell clones at the time of infection on day 0. White bars represent outgrowth in cultures of EBV-infected, autologous, mixed PBMCs. (C) AC clones fail to inhibit $CD23^+$ B-cell outgrowth. Equal numbers of the AC.E116 and AC.E1111 clones, the JB.flu10 clone, or mixed autologous $CD4^+$ T cells were added to 1.5×10^6 EBV-infected CD3-depleted PBMCs/ml from donor AC cells on day 0. The percent change which addition of T cells effected on $CD23^+$ B-cell outgrowth is listed to the right of the graphs as the percent regression.

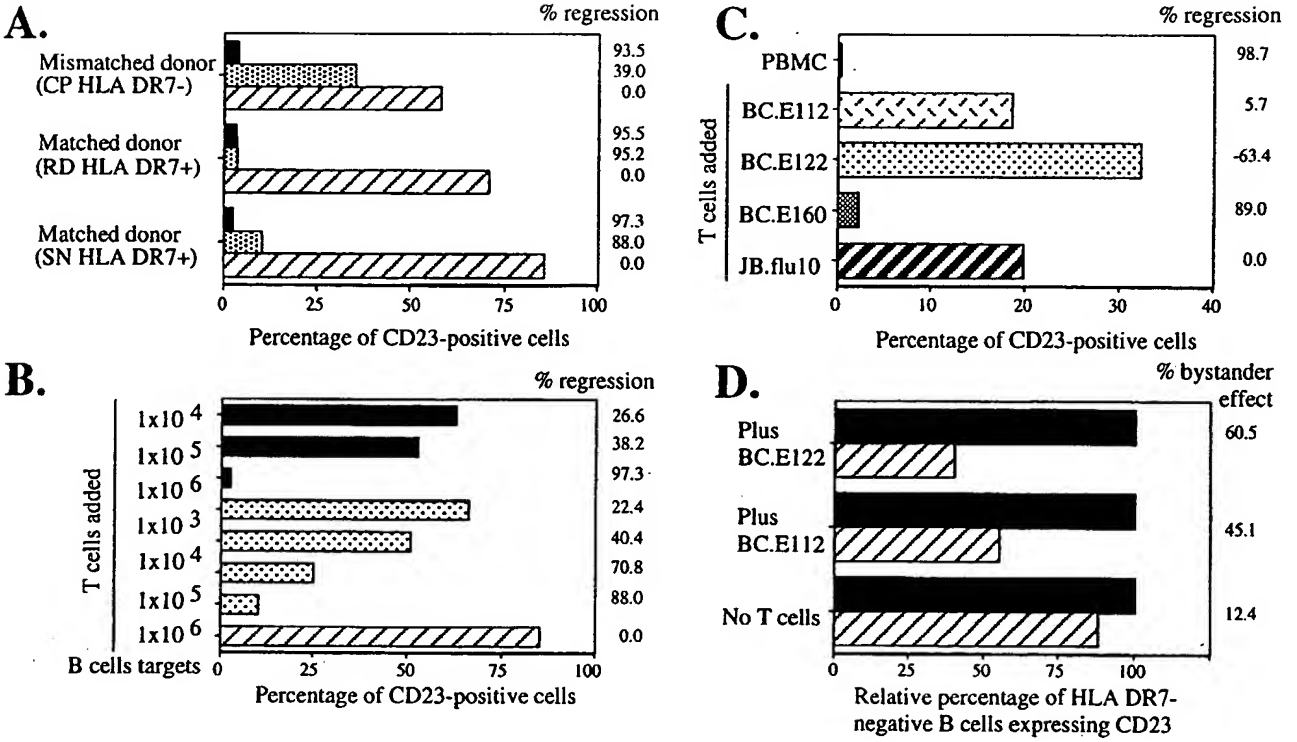


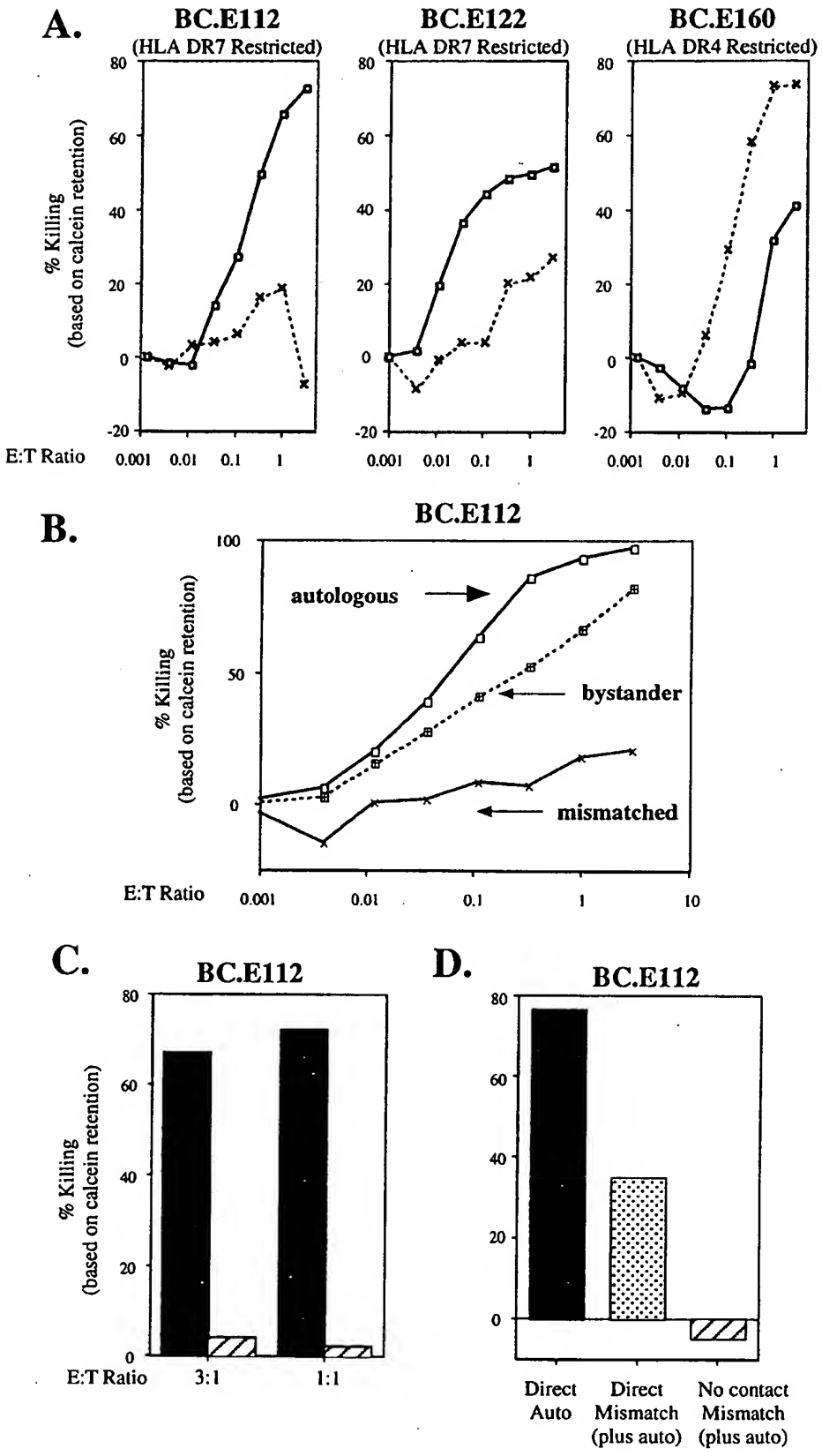
FIG. 6. EBNA1-reactive CD4+-T-cell clones inhibit outgrowth of CD23+ B cells in EBV-infected cultures derived from MHC II-matched donors and from mismatched bystander B cells. Shown is the percentage of CD23+ B cells remaining after 18 days in culture. (A and B) CD4+-T-cell clone BC.E112 inhibited outgrowth of EBV-infected B cells from HLA-DR7+ but not HLA-DR7− donors. Equal numbers of autologous mixed CD4+ cells (solid bars), clone BC.E112 cells (stippled bars), or no T cells (hatched bar) were added to 10^6 EBV-infected B-cell targets/ml derived from HLA-DR7+ or HLA-DR7− donors on day 0. (B) Tenfold dilutions of autologous mixed CD4+ T cells (solid bars) or the BC.E112 clone (stippled bars) were added to 10^6 EBV-infected B-cell targets/ml (hatched bars) from an HLA-DR7+ matched donor on day 0. (C) Clone BC.E160 inhibited outgrowth of EBV-infected B cells from an HLA-DR4+ donor. Equal numbers of the HLA-DR7-restricted BC.E112 and BC.E122 clones, the HLA-DR4-restricted BC.E160 clone, or the JB.flu10 clone were added to 1.5×10^6 EBV-infected HLA-DR4+ B-cell targets/ml on day 0. Cultures of mixed PBMCs derived from the HLA-DR4+ B-cell donor and seeded at 1.5×10^6/ml were cultured as well (solid bar). (D) Bystander inhibition of outgrowth of HLA-DR7-negative mismatched B cells. Equal numbers of the BC.E112 clone or the BC.E122 clone were added to 10^6 HLA-DR7-negative mismatched EBV-infected B cell targets/ml (solid bars) on day 0. Parallel cultures contained both mismatched and 10^6 HLA-DR7-positive matched B-cell targets/ml (hatched bars) in the presence of clone BC.E122, clone BC.E112, or no T cells. Mismatched HLA-DR7-negative targets were selectively analyzed. Comparison of the hatched bars to the solid bars demonstrates the extent of inhibition exerted over mismatched CD23+ B-cell targets due to the presence of matched HLA-DR7-positive targets. The percent change which addition of MHC-matched B cells effected on CD23+ B-cell outgrowth is listed to the right of the graphs as percent bystander effect.

gression. The AC clones had the same effect as an MHC-mismatched CD4+-T-cell clone that recognized influenza virus-infected cells (Fig. 5C). These results show that not all EBNA1-reactive CD4+-T-cell clones were competent to mediate regression and that secretion of low levels of IFN-γ in response to autologous LCLs correlated with poor performance in regression assays (Fig. 3).

EBNA1-reactive CD4+-T-cell clones enact regression on MHC II-matched, EBV-infected B-cell targets from multiple donors. We next investigated whether the BC clones could exert immune control over EBV-infected B cells from nonautologous, HLA-DR7-negative and -positive donors and from HLA-DR4-negative and -positive donors. Purified B cells were used in these experiments in order to avoid potential interactions of the CD4+ clones with natural killer cells that might be present in CD3-depleted target cell cultures. Addition of clone BC.E112 cells to HLA-DR7-positive matched B cells from two different donors resulted in regression. The addition of HLA-DR7-restricted BC.E112 or BC.E122 did not significantly impact the outgrowth of CD23+ B cells from HLA-DR7-negative donors (Fig. 6A and C). Similarly, the HLA-DR4-restricted BC.E160 clone exerted immune control over B cells from an HLA-DR4-positive donor, whereas the BC.E112, BC.E122, and JB.flu10 clones did not (Fig. 6C).

When mixed CD4+ T cells or clone BC.E112 was added to cultures at a T cell-to-B cell ratio of 1:1, the two sets of T cells were similar in their ability to cause regression (Fig. 6B, data for 10^6 T cells added/ml). However, when seeded at lower cell numbers, the clones were approximately 10-fold more effective in their ability to cause regression than the mixed CD4+ population: 10^5 mixed CD4+ T cells/ml enacted a degree of regression similar to that seen with 10^4 BC.E112 cells/ml.

EBNA1-reactive T-cell clones BC.E112 and BC.E122 enact "bystander" control over newly EBV-infected B cells from MHC II-mismatched donors. IFN-γ secretion (Fig. 3) and regression assays (Fig. 5 and 6A to C) demonstrated that only EBV-infected targets expressing a matched MHC II molecule could activate EBNA1-reactive clones. Once activated, how-


A.
BC.E112
(HLA DR7 Restricted)
BC.E122
(HLA DR7 Restricted)
BC.E160
(HLA DR4 Restricted)
% Killing
(based on calcein retention)
E:T Ratio
0.001
0.01
0.1
1
B.
BC.E112
autologous
bystander
mismatched
10
C.
BC.E112
3:1
1:1
D.
BC.E112
Direct
Auto
Direct
Mismatch
(plus auto)
No contact
Mismatch
(plus auto)

ever, the clones exerted their effects on targets that were not MHC matched (Fig. 6D). To demonstrate this phenomenon, B cells from an HLA-DR7-positive and an HLA-DR7-negative donor were exposed individually or in coculture to the HLA-DR7-restricted BC.E112 and BC.E122 clones. CD23 expression within the HLA-DR7-negative mismatched B-cell population was selectively analyzed. The addition of HLA-DR7-positive matched B cells in the presence of the BC.E112 or BC.E122 clone resulted in a 45 to 60% decrease in CD23 expression on the mismatched B cells. This decrease was of the same magnitude as the 42 to 47% decrease effected by the BC.E112 and BC.E122 clones on the CD23 expression of matched B cells in this same experiment (Fig. 6D and data not shown). These results indicate that once the EBNA1-reactive $CD4^+$ clones were activated by MHC II-matched EBV-infected B cells, MHC-mismatched EBV-infected B cells became targets for bystander regression.

EBNA1-reactive $CD4^+$-T-cell clones lyse MHC II-matched LCLs and mismatched bystander LCLs. IFN-γ secretion by the EBNA1-reactive $CD4^+$ clones in response to LCL targets was a marker of their ability to cause regression of newly EBV-infected B cells. Therefore, to elucidate potential mechanisms used by the EBNA1-reactive clones to exert regression, we investigated whether they possessed cytolytic activity against LCLs. In a calcein release assay, the BC.E112 clone caused losses of 70 and 72% of retained fluorescence from autologous and HLA-DR7-matched LCLs, respectively, at an effector-to-target (E:T) ratio of 3:1 (Fig. 7A and data not shown). The BC.E112 clone caused minimal background lysis of HLA-DR7-mismatched LCLs. BC.E122 and BC.E160 lysed MHC II-matched LCL targets with similar efficacy. The BC.E112 clone effected significant lysis of autologous LCLs during a short 5-h incubation; the other two BC clones required 18 h of incubation with the targets before killing became evident (data not shown).

In a phenomenon similar to that of bystander regression, the presence of MHC-matched targets rendered MHC-mismatched LCLs susceptible to bystander lysis by EBNA1-reactive clones (2). Incubation of dye-loaded autologous LCLs with a mismatched LCL did not alter the efficient lysis of the autologous LCL by BC.E112 (Fig. 7B). However, such coincubation resulted in lysis of dye-loaded mismatched LCLs that were not lysed when incubated with BC.E112 in the absence of the autologous LCL. Bystander killing of the mismatched LCL was less efficient than killing of the autologous LCL. At an E:T ratio of only 1:10, BC.E112 cells released 63% of dye from autologous LCLs; in contrast, BC.E112 cells were required at an E:T ratio of 1:1 to release dye from 66% of the mismatched bystander LCLs.

To investigate whether cytotoxicity could be mediated by soluble factors, such as IFNs or TNFs secreted into the culture supernatant, we performed calcein release experiments on BC clones and LCL targets in direct contact or on LCL targets separated from the activated clones by a porous membrane. The data displayed in Fig. 7C demonstrate lack of cytolysis in autologous LCL targets which were separated by a transwell membrane from autologous LCLs in direct contact with clone BC.E112. Similarly, MHC II-mismatched LCLs, which were subject to bystander lysis when in direct contact with autologous LCLs and clone BC.E112, were not lysed when they were separated by a membrane from activated T-cell cultures (Fig. 7D).

EBNA1-reactive $CD4^+$-T-cell clones do not use perforin to lyse LCLs. Classic mechanisms of contact-dependent T-cell-induced death include release of preformed vacuolar granzymes and perforin into an intracellular space and up-regulation on the cell surface of molecules like Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) (35, 58, 77, 82). These two effector pathways can be differentiated by the selective action of concanamycin A and brefeldin A (31, 33, 46, 79). Incubation with concanamycin A, which raises the pH in intracellular vacuoles and inactivates perforin, did not inhibit the killing of autologous LCLs by the BC.E112 or BC.E160 clone (Fig. 8A). In contrast, exposure of the clones to brefeldin A, which prevents progression of newly synthesized proteins through the Golgi apparatus, completely blocked the clones' ability to lyse autologous LCLs. Using the MS.B11 $CD8^+$-T-cell clone specific for EBNA3A, we verified that concanamycin A, but not brefeldin A, inhibited classical $CD8^+$-T-cell perforin-mediated killing of an antigen-loaded LCL in a 3-h calcein retention assay (Fig. 8B).

The inability of concanamycin A to inhibit the lytic activity of the $CD4^+$ clones correlated with their lack of perforin mRNA and protein. As detected by RPA, PMA and ionomycin up-regulated transcripts for granzymes A, B, and H but did not up-regulate perforin mRNA in the BC.E112 clone (data not shown). Similarly, antibodies that detected perforin in antigen-stimulated $CD8^+$-T-cell clone MS.B11 did not bind more avidly than an isotype control antibody to LCL-stimulated $CD4^+$ BC T-cell clones (Fig. 8C and data not shown).

Fas and Fas ligand contribute to lysis of LCLs by EBNA1-reactive $CD4^+$-T-cell clones. The inhibition of killing by addition of brefeldinA or by physical separation behind a membrane suggested that the $CD4^+$-T-cell clones employed a cell surface protein such as Fas ligand to effect LCL death. By RPA, we demonstrated that treatment of clone BC.E112 with PMA and ionomycin induced Fas ligand mRNA (Fig. 9A). Fas ligand protein was also up-regulated in the BC clones after

FIG. 7. EBNA1-reactive $CD4^+$-T-cell clones are cytotoxic following direct contact with MHC II-matched LCLs and with mismatched bystander LCLs. (A) Retention of calcein dye in MHC II-matched and -mismatched LCLs exposed for 19 h to each of three BC clones. LCL targets were HLA-$DR4^-$/$DR7^+$ (solid line, squares) or HLA-$DR4^+$/$DR7^-$ (dashed line, x's). (B) Retention of calcein dye 18 h after addition of the BC.E112 clone to autologous LCLs (solid line, squares), MHC-mismatched HLA-$DR7^-$ LCLs (solid lines, x's), or a combination of autologous and mismatched LCLs (dotted line, filled squares). In cultures where LCLs were combined, only the HLA-$DR7^-$ mismatched LCLs were labeled with calcein and analyzed. (C) Cytotoxicity measured by retention of calcein dye by autologous LCLs after 18 h of direct contact with the BC.E112 clone (solid bars) or by autologous LCLs separated from the same BC.E112-LCL cocultures by a porous membrane (hatched bars). (D) Cytotoxicity against autologous LCLs (solid bars) or against bystander MHC II-mismatched LCLs (stippled bars) after 18 h of direct contact with clone BC.E112 or against the same mismatched LCLs separated from BC.E112-autologous LCL cultures by a porous membrane (hatched bar).

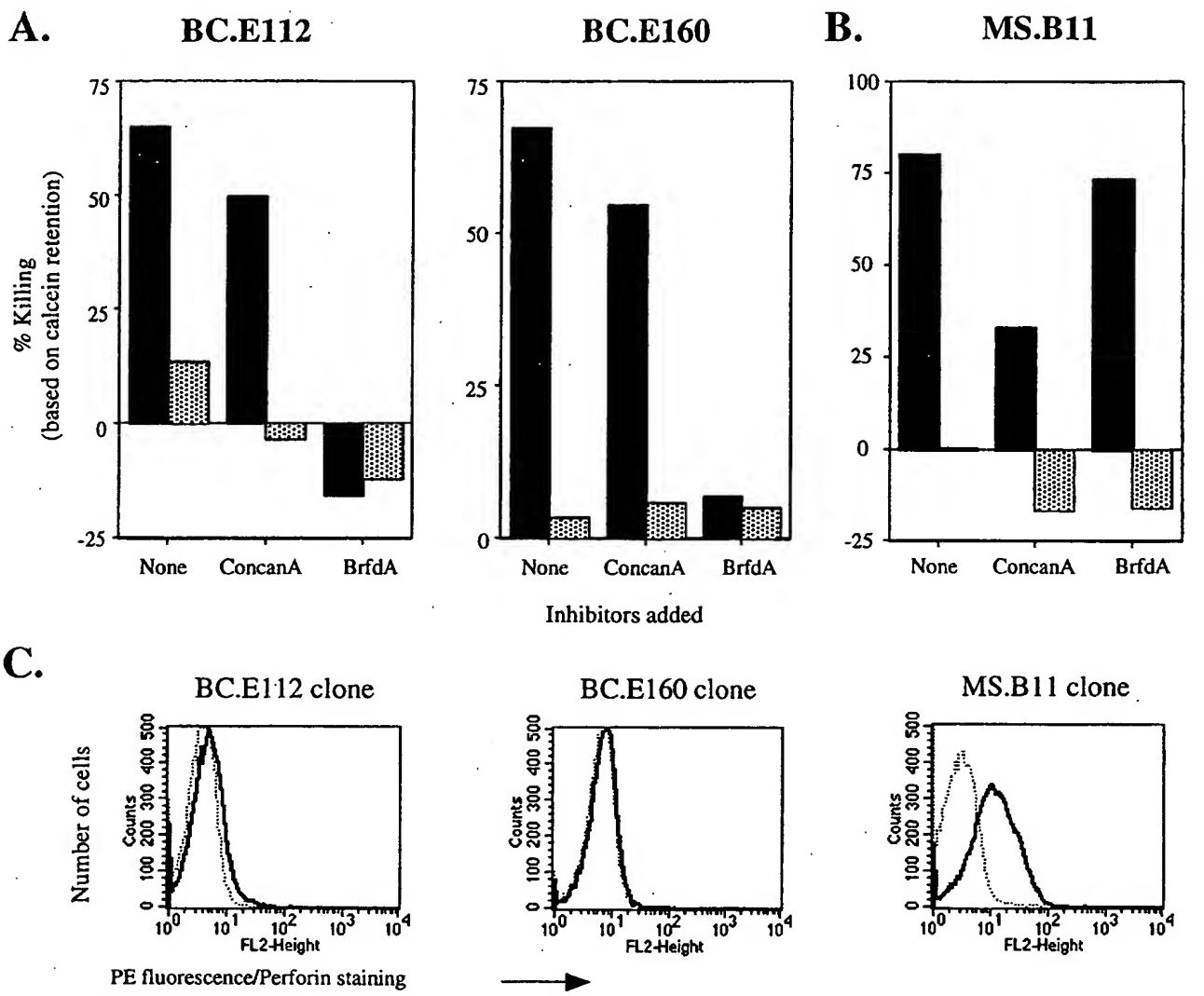


FIG. 8. EBNA1-reactive CD4$^+$-T-cell clones do not utilize perforin to induce cytolysis of LCLs. (A) Cytotoxicity against autologous (solid bars) or MHC II-mismatched (stippled bars) LCL targets after 18 h in culture with BC.E112 or BC.E160 cells at a 1:1 ratio. Prior to exposure to LCLs, clones were incubated with concanamycin A (ConcanA), brefeldin A (BrfdA), or culture medium alone (none). (B) Loss of calcein dye by MHC I-matched LCL targets with (solid bars) or without (stippled bars) addition of EBNA3A antigenic peptide after 3 h in culture with MS.B11 CD8$^+$-T-cell clones at a 1:1 ratio. (C) Intracellular staining with antibody to perforin. BC EBNA1-reactive CD4$^+$ clones were stimulated with autologous LCLs; the EBNA3A-specific CD8$^+$-T-cell clone MS.B11 was stimulated with peptide-loaded, MHC I-matched LCL (solid lines). Staining with a PE-conjugated isotype control is shown by the dotted lines.

incubation with PMA and ionomycin or with MHC II-matched LCLs (Fig. 9B and data not shown). In addition, the BC.E112 T-cell clone and the autologous LCL constitutively expressed Fas transcripts (Fig. 9A). All the CD4$^+$-T-cell clones and target LCLs expressed Fas protein on their surface (data not shown). Incubation with the ZB4 neutralizing antibody, which binds Fas and blocks binding of Fas ligand, prevented loss of calcein fluorescence when the autologous LCL was exposed to either the BC.E112 or the BC.E160 clone (Fig. 9C and data not shown). Exposure of T cells to the NOK-2 antibody against Fas ligand also inhibited killing, particularly by the BC.E160 clone. Combination of the NOK-2 and ZB4 antibodies completely prevented lysis of MHC-matched LCLs and of bystander MHC-mismatched LCLs incubated in the presence of matched targets. In contrast, lysis of antigen-loaded LCLs by the CD8$^+$ MS.B11 clone was minimally inhibited by a combination of NOK-2 and ZB4 antibodies (Fig. 9D). Preincubation of the LCLs with the pan-caspase inhibitor zVAD-fmk also eliminated lysis by the BC CD4$^+$-T-cell clones (13, 30) (Fig. 9C). Thus, the CD4$^+$ BC T-cell clones utilized Fas-Fas ligand interactions and not soluble factors or perforin to induce apoptosis in matched LCLs and mismatched bystander LCLs.

DISCUSSION

We extensively characterized a set of three CD4$^+$-T-cell clones reactive to EBNA1 that inhibit the activation and early outgrowth of human B lymphocytes following infection in vitro

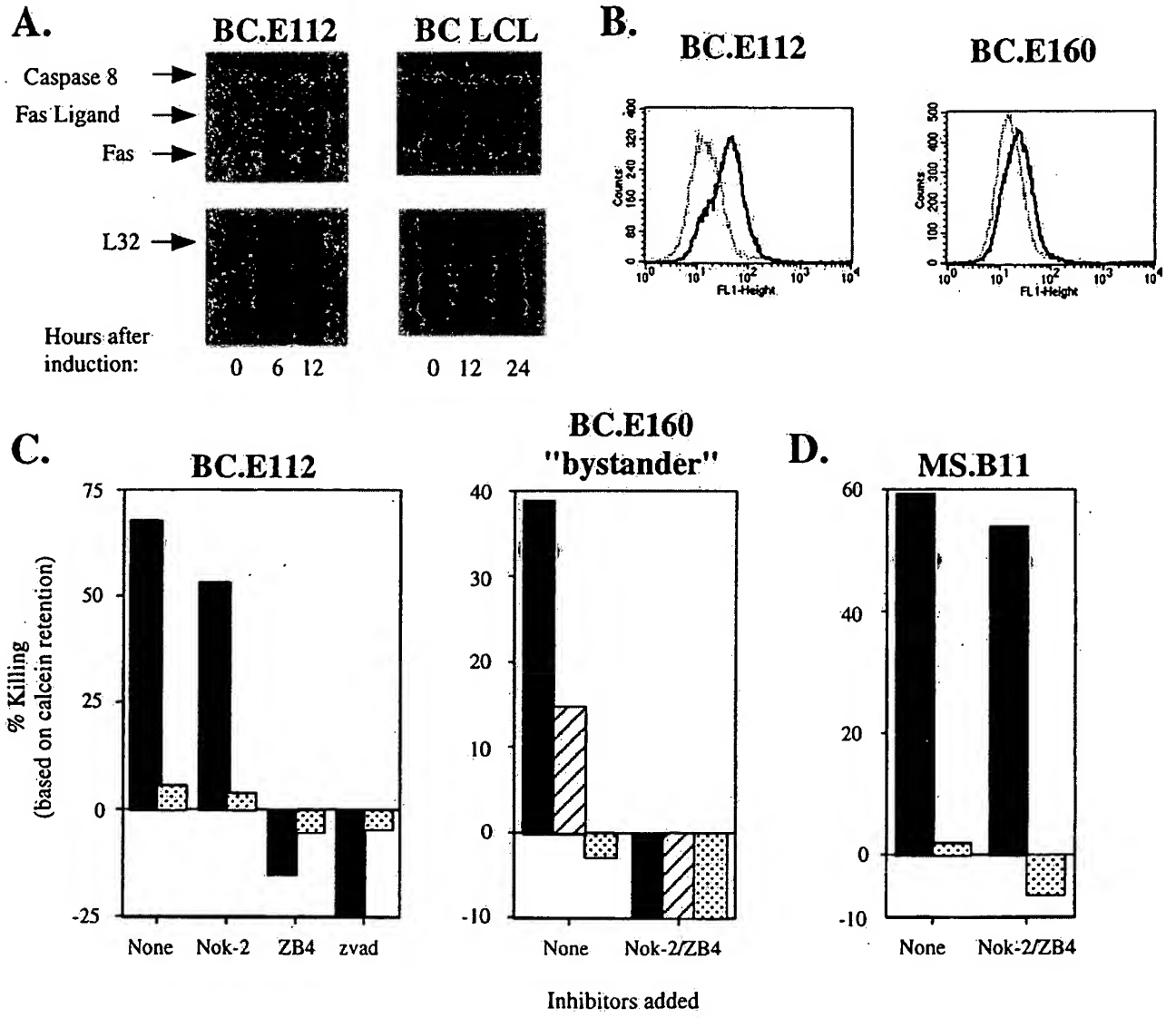


FIG. 9. EBNA1-reactive CD4+-T-cell clones express Fas ligand (FasL) when activated and induce cytolysis of LCLs via a Fas and FasL interaction. (A) RPA performed at the indicated times after stimulation of the BC.E112 clone or autologous LCLs with PMA and ionomycin. Relevant transcripts are indicated by arrows. L32 represents a housekeeping gene. (B) Intracellular staining with NOK-2 antibody to Fas ligand expressed by the BC clones after stimulation with autologous LCL (solid lines). Staining with an isotype control antibody and the secondary FITC-conjugated anti-murine IgG antibody is shown by the dotted lines. (C) Loss of intracellular calcein dye by autologous LCLs (solid bars) or MHC II-mismatched (stippled bars) LCL targets alone after 18 h in culture with BC.E112 clones (E:T ratio of 2:1) or BC.E160 clones (E:T ratio of 1:1). The panel entitled "BC.E160 bystander" compares killing of autologous LCLs (solid bars), MHC II-mismatched LCLs (stippled bars), and MHC II-mismatched LCLs in the presence of autologous LCLs (hatched bars). (D) Loss of calcein dye by MHC I-matched, antigen-loaded (solid bars) or nonloaded (stippled bars) LCLs after 3 h in culture with CD8+-T-cell clone MS.B11 at an E:T ratio of 1:1. In panels C and D, T cells and LCLs were incubated with culture medium (none), the NOK-2 antibody to Fas Ligand, the ZB4 neutralizing antibody to Fas, a combination of both antibodies (NOK-2/ZB4), or the pan-caspase inhibitor zVAD-fmk.

with EBV. These results contribute to the growing list of antiviral activities mediated by CD4+ T cells and establish the principle that CD4+ T cells directed against one viral protein are competent to mediate early immune control over EBV-induced B-cell outgrowth. The clones are cytotoxic to autologous and MHC II-matched lymphoblastoid cells. Cytotoxicity requires cell-to-cell contact and is mediated by Fas/Fas ligand interactions. In both regression and cytotoxicity assays, the CD4+-T-cell clones exerted bystander effects in which MHC II-mismatched B-cell targets were inhibited from outgrowth or lysed, but only in the presence of MHC II-matched B-cell targets. Cytotoxic CD4+ T cells directed against EBNA1 may assume a role in T-cell immunoprophylaxis or immunotherapy of B-cell LPDs. They are of particular importance since EBNA1 is expressed in every EBV-associated malignancy and is not well recognized by CD8+ T cells (8, 37, 68).

Characterization of cytotoxic CD4+-T-cell clones reactive to EBNA1. Three CD4+-T-cell clones were isolated from a single

individual (BC line) by virtue of their capacity to secrete IFN-γ following alternate exposure to autologous DCs infected with a vaccinia virus vector expressing EBNA1 and autologous EBV-transformed lymphoblastoid cells. The published literature contains contradictions as to whether Th1 or Th2 cytokines dominate the responses of mixed $CD4^+$ T cells to EBNA1 (7, 61, 83). Our novel finding is that BC clones expressed cytokines of both Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10, and IL-13) types following application of T-cell activation stimuli (Fig. 2). BC.E160 secreted large amounts of IL-4 and IL-5, whereas the cytokine responses of BC.E112 and BC.E122 were skewed toward Th1-type cytokines. Since the BC.E160 clone expressed both Th1 and Th2 cytokines in the same cell, it can be classified as Th0. All three clones caused regression and cytolysis to the same extent (Fig. 5 to 9). Neither of these functional effects could be reproduced by $CD4^+$-T-cell clones separated from targets by membrane filters. Therefore, while cytokine expression reflects the differentiation and activation state of the clones and helps to classify them, the secreted cytokines themselves are not likely to be essential effector mechanisms.

Inhibition of proliferation of EBV-infected B cells by $CD4^+$-T-cell clones reactive to EBNA1. The capacity of the clones to exert regression was assessed with our newly developed techniques which measure early outgrowth of CD23-positive B cells (57, 60). The number and percentage of $CD23^+$ B cells have been shown to correlate with the number of EBNA-positive, EBV-infected cells detected by immunofluorescence in cultures maintained for 2 weeks after exposure in vitro to EBV. Cell-mediated immunity as measured by early outgrowth of $CD23^+$ B cells and immunity measured by longer-term outgrowth assays both exhibit a dependence on T cells, a sensitivity to immunosuppressive drugs, a dependence on initial cell density, and a lack of dependence on macrophages (57, 69). Classical outgrowth assays have always emphasized the role of $CD8^+$ T cells (41). However, by assaying for $CD23^+$ B-cell outgrowth at 2 to 3 weeks after infection, we elucidated a role for $CD4^+$ T cells in immune control over EBV (57). Therefore, we chose to measure $CD23^+$ B-cell percentages to investigate the effector functions of $CD4^+$ T cells reactive to EBNA1.

When added to freshly infected B cells, even at a ratio of 1 T cell to 100 B-cell targets, the three BC clones markedly reduced the outgrowth of $CD23^+$ B cells (Fig. 5 and 6). The specificity of regression mediated by the $CD4^+$-T-cell clones was established in several ways: (i) at early times after infection, three- to fourfold more clones responded to EBV-infected B cells by secretion of IFN-γ than reacted to mock-infected B cells (Fig. 4). (ii) $CD4^+$-T-cell clones matched for MHC II enacted regression, whereas those mismatched for MHC II did not (Fig. 6A and C). (iii) A $CD4^+$-T-cell clone raised to a non-EBV antigen, i.e., proteins from influenza virus, did not react to EBV-infected B cells (Fig. 4B), nor did it inhibit outgrowth of $CD23^+$ B cells following EBV infection (Fig. 6C).

At an E:T ratio of 1:1, mixed $CD4^+$ T cells were slightly more efficient inhibitors of B-cell proliferation than were the $CD4^+$-T-cell clones (Fig. 6A and B). However, the BC.E112 clone markedly inhibited B-cell proliferation at an E:T ratio of 1:10, whereas the mixed cells were effective only at an E:T ratio of 1:1. Thus, the clonal population was 10-fold more active than autologous mixed $CD4^+$ T cells. Since the number of EBV antigen-specific T cells present in the mixed $CD4^+$-T-cell population is likely to be significantly lower than in the clonal population, their somewhat greater activity at high E:T ratios suggests three hypotheses that need to be explored in future experiments: (i) The mixed population is likely to include $CD4^+$ T cells that recognize EBV antigens other than EBNA1. The other antigens may provide stronger stimulation. (ii) The mixed $CD4^+$-T-cell population may include cells, reactive to EBNA1 or other EBV antigens, with very-high-affinity T-cell antigen receptors. These high-affinity T cells may not have been cloned. (iii) The EBV-specific mixed $CD4^+$-T-cell population may employ more-potent effector mechanisms or may expand more rapidly to control B-cell outgrowth than do the clones.

Cytotoxic activity of EBNA1-reactive clones. A number of mechanisms could account for the capacity of the BC clones to inhibit proliferation of $CD23^+$ B cells following EBV infection. These include down-regulation of CD23 from the surface of B cells, noncytotoxic inhibition of proliferation of EBV-infected $CD23^+$ B cells, and B-cell lysis during virus reactivation and release, as well as classical lysis (17). Modes of lysis employed by various EBV-specific $CD4^+$ T cells include release of perforin and granzymes, granulysin release, and up-regulation of surface Fas ligand and TRAIL molecules (85, 86, 93, 94). The ability of brefeldin A to block cytotoxicity by the BC clones, the expression of Fas ligand on the surface of activated clones and Fas on target cells, and the neutralizing effect of antibodies to Fas and Fas ligand all support the hypothesis that the mechanism of cytotoxicity of the BC clones was apoptosis initiated by Fas and Fas ligand interactions.

Our experiments appear to exclude contributions to cytolytic activity of BC clones by several mechanisms other than Fas and Fas ligand interactions. Cytotoxicity mediated by a perforin-granzyme mechanism was excluded by showing that concanamycin A, an inhibitor of vacuolar proton pumps which leads to perforin degradation, did not block cytotoxicity of the BC clones towards MHC II-matched target cells (Fig. 8A) (33). Moreover, the $CD4^+$-T-cell clones did not express perforin protein upon activation, whereas a cytotoxic $CD8^+$-T-cell clone did (Fig. 8C). Cytotoxicity did not appear to be mediated by a soluble cytokine. Transfer of supernatants from mixed cultures of $CD4^+$-T-cell clones and matched LCLs to a fresh LCL did not transfer lytic activity (data not shown). LCL cells separated by a membrane from mixtures of $CD4^+$-T-cell clones and activating LCLs were not lysed (Fig. 7 and 9). Since our data demonstrate that the clones are cytotoxic to LCL through Fas and Fas ligand interactions, it seems likely that a similar mechanism is responsible for their activity in the regression assay against freshly EBV-infected B cells.

Bystander effect. We suggest a plausible mechanism for the bystander effect seen in early regression and cytotoxicity assays. A T-cell clone activated by encounter with an MHC II-matched target up-regulates Fas ligand on its surface. Upon interaction with a susceptible mismatched cell that expresses Fas, the T-cell clone would be cytotoxic. The physical interaction of an activated clone with a mismatched target would be less likely to occur, and the interaction would not be augmented or sustained by cognate interactions between the T-cell antigen receptor and MHC II loaded with antigenic peptide.

Therefore, bystander cytotoxic effects would be less efficient than MHC II-matched interactions. This model gains support from our experimental observations that antibodies to Fas and Fas ligand also inhibit the bystander reaction (Fig. 9C). Moreover, bystander cytotoxicity effects also required cell-to-cell contact: MHC II-mismatched LCL cells separated by a membrane from a mixture of $CD4^+$-T-cell clones and MHC II-matched LCLs were not lysed.

Some EBNA1-reactive $CD4^+$-T-cell clones do not mediate regression. $CD4^+$-T-cell clones are often generated by exposure to EBNA1 peptides (37, 43). We studied two EBNA1-reactive $CD4^+$-T-cell clones derived from a single individual (AC line) by exposure to autologous DCs pulsed with an EBNA1 peptide (aa 514 to 527) that was known to be presented by the HLA-DR1 molecule (37). The AC clones could not mediate regression; their lack of activity in the regression assay was similar to a $CD4^+$-T-cell clone directed against influenza virus (Fig. 5C). Although the AC clones secreted copious amounts of IFN-γ in response to autologous DCs loaded with recombinant EBNA1 protein (Fig. 1B), their response to MHC II-matched LCLs was 300 times weaker than that of BC clones exposed to comparable targets. Remarkably, the AC clones failed to secrete IFN-γ when exposed to the autologous LCL (Fig. 3A and data not shown). These results suggest that $CD4^+$-T-cell clones, such as AC, that do not secrete cytokines in response to the autologous LCL are not likely to inhibit outgrowth of freshly infected B cells.

Further work is needed to explore reasons for the dramatic differences in behavior between the BC and AC clones. One explanation is that the EBNA1 peptide (aa 514 to 527) antigen recognized by the AC clones was not efficiently generated or presented by MHC II molecules on LCL. The poor, or even absent, response of the AC clones to MHC II-matched LCL is consistent with this explanation. Another related explanation may lie in the relatively low affinity of the T-cell antigen receptor on the AC clones. The T-cell receptor affinity may be low for any HLA-DR1 molecule containing the EBNA1 aa 514 to 527 peptide, even MHC complexes loaded exogenously, or only for the HLA-DR1/EBNA1 aa 514 to 527 complex which is selected by intracellular processing of EBNA1 (65). Support for these hypotheses stems from our preliminary experiments indicating that the AC.E116 clone secreted IFN-γ and lysed autologous LCLs after they were pulsed with large amounts of the EBNA1 aa 514 to 527 peptide (data not shown).

The AC clones were derived by exposure to high levels of peptide loaded on the surface of DCs, whereas the BC clones were selected for their response to more physiologic levels of EBNA1 antigen presented by vaccinia virus-infected DCs and by LCLs. The second method of generation may lead to isolation of clones with higher-affinity T-cell antigen receptors. In studies of $CD8^+$ T cells reactive to melanoma antigens, sorting for populations that exhibit high levels of tetramer binding has proven useful for isolation of cytotoxic $CD8^+$-T-cell clones (95). Such a T-cell receptor affinity selection step or exposure to targets with physiological levels of antigen, such as LCLs, may be necessary to raise clones capable of preventing EBV-induced B-cell outgrowth. Our findings suggest that heterogeneity exists in the antigen affinity of polyclonal EBNA1-reactive $CD4^+$-T-cell populations. This heterogeneity could result from T-cell priming in vivo by an antigen-presenting cell other than an LCL or EBV-infected B cell. High- or low-affinity clones may be preferentially expanded, depending on the stimulation conditions.

Potential clinical applications of EBNA1-reactive $CD4^+$-T-cell clones. Progression to transformation and establishment of immortal EBV-positive cell lines in vitro correlates with expansion of $CD23^+$ B cells; similarly, the progression to EBV-associated LPD is characterized by infection of new B-cell targets and malignant expansion of the $CD23^+$-B-cell population. An EBNA1-reactive $CD4^+$-T-cell clone, which has been shown by in vitro experiments such as we describe to be competent to prevent early outgrowth of $CD23^+$ B cells newly infected with EBV (Fig. 5 and 6), may prove useful in preventing $CD23^+$ EBV^+ B-cell lymphomas in vivo. A panel of such $CD4^+$-T-cell clones might be raised to recognize EBNA1 presented on different MHC II molecules. The appropriate $CD4^+$-T-cell clone could be administered to patients with the corresponding MHC II allele. In the instance of donor lymphomas arising in recipients of allogeneic T-cell-depleted bone marrow or stem cell transplants, the $CD4^+$-T-cell clone would be matched to the donor. In the case of posttransplant LPD (PTLD) arising following solid organ transplantation, the $CD4^+$-T-cell clone would be matched to the recipient (23, 47). This strategy might be more efficient than the current, time-intensive approach of adoptive immunoprophylaxis and therapy of PTLD, which involves raising LCLs and subsequently polyclonal EBV-reactive T-cell lines for each individual.

When $CD4^+$-T-cell clones directed against EBNA1 are used clinically, it will be essential to determine whether the bystander cytotoxicity that these clones exhibit in vitro will have beneficial or harmful effects. As shown in Fig. 7, clones which had been activated by EBNA1-expressing MHC II-matched targets exerted cytotoxic effects in vitro against targets that were in direct contact with the T cells but were not MHC II matched. Clones would be activated in vivo by EBV-positive, EBNA1-expressing cells. Bystander activity might augment control over other cells in the immediate area, which might contribute to lymphoma tumor burden. For example, B cells within the lymphoma population which present EBNA1 antigen inefficiently but are stimulated to express Fas upon EBV infection would be subject to bystander killing even if those cells alone would not have stimulated T-cell activity. On the other hand, bystander reactions may be a potential source of unwanted toxicity by the clones, particularly if the T cells remain activated even after they have migrated away from the EBV-infected tumor cells which provided the initial stimulus.

The approach of using well-characterized T-cell clones in immunotherapy and immunoprophylaxis need not be limited to $CD4^+$-T-cell clones directed against EBNA1. T cells reactive towards many viral antigens are primed during primary infection and likely help prevent the development of EBV-associated malignancies in healthy carriers. EBNA1-reactive clones might be given in combination with $CD4^+$-T-cell clones directed against EBNA2, LMP2a, or lytic cycle antigens, such as BHRF1 (60, 85, 88, 92). These could be mixed with cytotoxic $CD8^+$-T-cell clones specific for the immunodominant EBNA3A, EBNA3C, or LMP2 proteins. Such comprehensive immunotherapy would not be limited to LPDs but might be applied to other EBV malignancies, such as Hodgkin's disease and BL (61, 73).

Since EBNA1 is the only viral protein consistently expressed in all forms of viral infection and is not well recognized by $CD8^+$ T cells, the potential contribution of EBNA1-reactive $CD4^+$-T-cell clones to adoptive immunotherapy regimens against PTLD and many EBV-associated tumors merits further investigation.

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